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DEDICATED

To my loving parents,

My wife Mitra and sweet girl Nika

Abbreviations:

ACF: Aberrant Crypt Foci

AOX: Antioxidant

BMI: Body Mass Index

BP: Blood Pressure

BSA: Bovin Serum Albumin

CBC: Cell Blood Count

CVD: Cardiovascular disease

DNP: Dinitrophenyl

EPR: Electron Paramagnetic Resonance

GDP: Gross Domestic Product

GPx: Glutathione Peroxidase

GR: Glutathione Reductase

H_2O_2: Hydrogen peroxide

Hb: Hemoglobin

HRP: Horseradish peroxidase

ID: Iron Deficiency

IDA: Iron Deficiency Anemia

IPAQ: International Physical Activities Questionnaire

IQR: Inter quartile range

MDA: Malondialdehyde

MT: Metric Ton

MNM: Micronutrient Malnutrition

NIMS: National Integrated Micronutrient Survey

Ngr: Nano gram

NMR: Nuclear Magnetic Resonance

NNFTRI: National Nutrition & Food Technology Research Institute

O_2° : Superoxide

$OH^{\cdot}$: Hydroxyl radical

OS: Oxidative stress

OSS: oxidative stress score

OxLDL: Oxidized LDL

PC: Protein carbonyls

PHC: Primary Health Care

RAE: Retinol Activity Equivalent

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SF: Serum ferritin

SOD: Supper Oxide Dismutase

STfRs: Serum Transferrin Receptors

TAC: Total Antioxidant Capacity

TIBC: Total Iron Binding Capacity

UNICEF: United Nation Children Fund

USDA: United States Department of Agriculture

WB: World Bank

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1 INTRODUCTION

Micronutrient deficiencies are very common in developing regions of the world although they are seen in industrialized nations as well. It affects all age groups but young children and women of reproductive age tend to be at the highest risk of developing micronutrient deficiencies. Worldwide, the three most common forms of micronutrient malnutrition are iron, vitamin A, and iodine deficiencies. It is estimated that over 2 billion people are anemic, nearly 2 billion have inadequate iodine nutrition, and 254 million preschool-aged children are vitamin A deficient. Even moderate levels of these deficiencies can have serious detrimental effects on human function (World Health Organization, 2000).

The high prevalence of Iron deficiency and its subsequent anemia in different age groups of most provinces of Iran has been shown in two studies of "National Integrated Micronutrient Survey (NIMS report, 2003) 2000" and "National Food Consumption Survey-2001"(NNFTRI report, 2005).

In spite of implementing iron supplementation and nutrition education, as well as public health measures through the Primary Health Care (PHC) system, Iron deficiency and its anemia is still one of the most common nutritional problems in Islamic republic of Iran. So, implementing an appropriate intervention for combating this public health problem of the country has been regarded as a priority of Iran's Ministry of Health & Medical Education (MOH&ME). In order to find a suitable, scientific and feasible solution and also to use other countries' experiences, an international workshop was held on this issue in Iran in 1996 (WHO/EMRO report, 1996). At the end of the workshop, some recommendations and solutions were presented to overcome the problem. One of the most important suggested solutions was food fortification strategy. Fortification of food with micronutrients is a valid technology for reducing micronutrient deficiency as part of a food-based approach whenever and wherever existing food supplies fail to provide adequate levels of certain nutrients in the diet. In such circumstances, food fortification reinforces and supports ongoing nutrition improvement programs and should be regarded as part of a broader, integrated approach to prevent micronutrient malnutrition. Fortification of foods with micronutrients is a technologically, programmatically,

and economically effective method of increasing micronutrients intake in populations (Nestel and Nalubola, 2000) (Lotfi, 2002) (Darnton-Hill and Nalubola, 2002).

It is suggested that food fortification might be an inexpensive, simple, and effective way of controlling and preventing iron deficiency and its related anemia in many countries (Allen et al, 2006). Since iron deficiency is one of the main public problems in Iran, Ministry of Health and Medical Education (MOH&ME) started technical studies on this subject. So, based on dietary information indicating that bread (Mean consumption in Iran 320gr/day) is an ideal vehicle for a fortification program in Iran, a pilot study of flour fortification with 30 mg/kg iron and 1.5 mg/kg folic acid was conducted in Bushehr province, in 2000. This intervention was conducted to evaluate its possible problems and capacities for extending it to a national level. In this way, the program gradually progressed and in 2008, national flour fortification was started as a mandatory program in the country. In all flour fortification programs, a mixture of micronutrients (Premix) is added to food with a special instrument (Microfeeder). In Iran, premix is produced inside the country and includes 42% ferrous sulphate, 0.75% folic acid, and 57.25% Maize starch. The additional rate is 200gr/MT.

Despite the advantages of this program, questions were raised by some nutritionists of steering committees and faculties from the beginning of the intervention about potential hazards of adding iron to flour for those who do not suffer from anaemia or iron deficiency. There were many reasons for their concern, which are summarized as follows:

1. Considering the natural amount of iron in flour, and also added iron through fortification (30 mg/kg as ferrous sulphate), the total amount of iron of flour after fortification is about 40-80 mg/kg. This amount of iron is about two times more than what we expect from the fortification program.

2. There is a wide range of daily intake of iron in the community. As the National Food Consumption Survey showed, the variety of iron intake among different age and sex groups in the country is from 3.6 to 68.9 mg/day (NNFTRI report, 2005).

3. Even in developing countries, parts of the community with moderate and high socio-economical background have an adequate or high intake of heme iron that is easily absorbed and also can interfere with the absorption of non-heme iron in the diet. This can lead to iron overload or other kinds of related problems (Schuemann, 2001).

Moreover, determining the tolerable upper intake level of iron is difficult, partly because of the fact that absorption of heme iron is much higher than non-heme iron and also the bioavailability of non-heme iron varies and can be influenced by different dietary factors.

However, Iron is crucially important for normal functioning of the human body; both iron overload and iron deficiency lead to severe diseases (Swanson, 2003). Iron overload is less common than iron deficiency, but can result in serious medical problems (Burke et al, 2001).

Absorption of iron in the body is a well controlled process. In iron deficiency homeostatic mechanisms increase intestinal iron absorption, but its down-regulation at high intake levels seems insufficient to prevent accumulation of high iron stores (Salonen et al, 1993) (Swanson, 2003). Moreover, iron is known to catalyze the generation of hydroxyl radicals from super oxide anions and to increase oxidative stress which in turn increases free iron concentration (Fang et al, 2002). Meanwhile, some animal experiments showed the negative effects of iron overload and oxidative damages (Knuston et al, 2000) (Day et al, 2003); However, the relevance of these studies to humans is unknown. Unfortunately, there are not enough human studies about the effects of high iron intake on oxidative damages to help us to make the right decision (Binkoski et al, 2004). So, in fortification programs with iron, concerns exist regarding iron over load in some parts of the community that are not anemic.

Today, the problems of oxidative stress and the effects of their incidence have been highly addressed by the scientific community. This is justified by the fact that oxidative stress plays an important role in the pathogenesis of many noncommunicable diseases and cellular defects that lead to specific diseases (Salonen et al, 1993) (Kurtoglu et al, 2003) (Sareen et al, 2003). So, despite the fact that Iran's flour fortification program just provides about 40% of the average

daily needs of the several population (based on the results of national food consumption survey, iron daily needs is 19 mg), and could not be dangerous even for non-anemic members of the community, regarding the importance of issue and the high number of non anemic population, this research was design. The aim of this research was to study the effects of flour fortification program on oxidative stress biomarkers and Iron status of non-anemic individuals, during two time periods (32 and 64 weeks). It should be noted that we could not find a similar study in the ltrature.

2 GOAL AND OBJECTIVES

2-1 Goal

The goal of this study was to investigate the effect of consuming fortified flour with iron, on oxidative stress biomarkers and iron status of non- anemic, apparently healthy, 40- to 65-year-old adults.

This study had two parts; the first part was a randomized field trial, and the second one was a before and after study.

2-2 Objectives

2-2-1Objectives of the First Part of the Study (Randomized Field Trial):

1- Determining the effect of consuming fortified flour with iron on iron status including hemoglobin, ferritin, serum iron, total iron binding capacity, transferrin saturation, and serum transferrin receptor of 40- to 65-year-old males and females in the case group, and comparing it with the control group at baseline and after 32 weeks.

2- Determining the mean values of oxidative stress biomarkers including total anti-oxidant capacity (TAC), malonedialdehyde (MDA), glutathione peroxidase (GPx), super-oxide dismutase (SOD), protein carbonyl (PC), and oxidized LDL of 40-65 years old males and females in case group and comparing it with the control group at baseline and after 32 weeks.

3- Determining daily intake of Iron, antioxidant vitamins (Vitamin A, C, E, and carotenoids) and other related biochemical parameters (albumin, total protein, and uric acid) in 40- to 65-year-old males and females in the case group and comparing it with the control group at baseline and after 32 weeks.

2-2-2 Objectives of the Second Part of the Study (Before and After Study):

1- Determining and comparing the effect of consuming fortified flour with iron on iron status of 40- to 65-year-old males and females at baseline and after 32 and 64 weeks in Semnan.

2- Determining and comparing the mean values of oxidative stress biomarkers of 40- to 65-year-old males and females at baseline and after 32 and 64 weeks in Semnan.

3- Determining daily intake of Iron, antioxidant vitamins and other related biochemical parameters in 40- to 65-year-old males and females at baseline, after 32 and 64 weeks in Semnan

2-3 Practical Objectives

The results of this study can help policy makers in designing future interventions based on food fortification in the country.

2-4 Impact

Enhancing health status in the community

2-5 Hypothesis of the Survey

1- Consuming Iron fortified flour does not cause iron overload in the study population after 32 and 64 weeks

2- Consuming Iron fortified flour does not induce oxidative stress biomarkers in the study population after 32 and 64 weeks

3 LITERATURE REVIEW

3-1 Micronutrient Deficiency

3-1-1 Micronutrient Deficiency in the World

Vitamins and minerals, often called micronutrients, are essential for human physical and mental health and survival. Only very small quantities of them are needed, but deficiencies can have disproportionately large, often life-threatening, effects (Verster, 2004). Yet deficiencies of many important vitamins, such as vitamin A and D, and minerals such as iron, zinc and iodine are widespread in many parts of the world (Verster, 1996). More than 2 billion people in the world today, nearly all of them in developing countries, suffer from nutritional deficiencies, mainly the lack of micronutrients such as iron, vitamin A, iodine and folic acid (World Health Report, 2002).

Iron deficiency (ID) is the most common micronutrient deficiency. The most commonly affected populations in developing countries are pregnant women, school-age children, non-pregnant women, and preschool children; as many as 50% of the elderly and adult males can be anemic as well. Factors other than ID that cause anemia include deficiencies of nutrients such as B12, folic acid, B6, riboflavin, vitamin A, and general infections and chronic diseases such as HIV/AIDS. Anemia has important health and economic consequences. A study of 15 countries showed that the median value of productivity losses due to ID was about USD 4/person/year or 0.9% gross domestic product (GDP), and this excluded the lowered effectiveness of money spent on education (World Bank, 1994). The World Bank has estimated that interventions to end micronutrient malnutrition were among the most cost-effective investments in the health sector. Failure to act in a country where micronutrient malnutrition exists can waste as much as 2-3% of the gross domestic product (GDP) (UNICEF, 2003), whereas acting to solve the problem comprehensively and sustainably would cost less than 0.3% of GDP.

In Iran, iron-deficiency anemia is one of the most common nutritional problems. A national survey conducted by the Ministry of Health and Medical Education and UNICEF in 1999 showed that 33.4% of the Iranian women were anemic according to hemoglobin levels, and 34.5% were iron deficient according to

serum ferritin levels. More than 38% of 15- to 23-month-old children were anemic as well. Among adolescents, data showed that the prevalence of anemia in different provinces was from 11% to 36 %. Iron deficiency is considered to be more common among adolescent girls than boys (MOH&ME, 2003).

Based on these findings, in Iran, it seems that iron deficiency and the resultant anemia are important nutritional problems.

3-1-2 Major Issues in Controlling Iron Deficiency

There are four main strategies for combating iron deficiency: food diversification, supplementation, food fortification, and public health measures (e.g. controlling parasitic infestations). In other words, iron status may be improved through food-based strategies (food diversification and food fortification) and nonfood-based strategies (supplementation and controlling parasitic diseases):

3-1-2-1 Food Diversification

Increasing dietary diversity means increasing both the quantity and the range of micronutrient-rich foods consumed. In practice, this requires the implementation of programs that improve the availability and consumption of, and access to, different types of micronutrient-rich foods (such as animal products, fruits and vegetables) in adequate quantities, especially among those who at risk for, or vulnerable to, micronutrient malnutrition (MNM). In poorer communities, attention also needs to be paid to ensuring that dietary intakes of oils and fats are adequate for enhancing the absorption of the limited supplies of micronutrients. Increasing dietary diversity is the preferred way of improving the nutrition of a population because it has the potential to improve the intake of many food constituents – not just micronutrients – simultaneously (Allen et al, 2006). Ongoing research suggests that micronutrient-rich foods also provide a range of antioxidants and probiotic substances that are important for protection against selected noncommunicable diseases and for enhancing immune function. However, as a strategy for combating MNM, increasing dietary diversity is not without its limitations, the main one being the need for behavior

change and for education about how certain foods provide essential micronutrients and other nutritive substances. A lack of resources for producing and purchasing higher quality foods can sometimes present a barrier to achieving greater dietary diversity, especially in the case of poorer populations.

3-1-2-2 Supplementation

Supplementation is the term used to describe the provision of relatively large doses of micronutrients, usually in the form of pills, capsules or syrups. It has the advantage of being capable of supplying an optimal amount of a specific nutrient or nutrients, in a highly absorbable form, and is often the fastest way to control deficiency in individuals or population groups that have been identified as being deficient.

In developing countries, supplementation programs have been widely used to provide iron and folic acid to pregnant women, and vitamin A to infants, children under 5 years of age and postpartum women. Because a single high-dose vitamin A supplement improves vitamin A stores for about 4–6 months, supplementation two or three times a year is usually adequate. However, in the case of the more water-soluble vitamins and minerals, supplements need to be consumed more frequently. Supplementation usually requires the procurement and purchase of micronutrients in a relatively expensive pre-packaged form, an effective distribution system and a high degree of consumer compliance (especially if supplements need to be consumed on a long-term basis). A lack of supplies and poor compliance are consistently reported by many supplementation program managers as being the main barriers to success (Allen et al, 2006).

3-1-2-3 Public Health Measures

In addition to the specific interventions outlined above, public health measures of a more general nature are often required to help prevent and correct MNM, because it is often associated with poor overall nutritional status and with a high prevalence of infection. Such measures include infection control (e.g. immunization, malaria and parasite control), and improvement of water and sanitation. Other factors, such as the quality of child care and maternal

education, also need to be taken into consideration when developing public health responses to MNM.

3-1-2-4 Food Fortification

Food fortification refers to the addition of micronutrients to processed foods. In many situations, this strategy can lead to relatively rapid improvements in the micronutrient status of a population, and at a very reasonable cost, especially if advantage can be taken of existing technology and local distribution networks. Since the benefits are potentially large, food fortification can be a very cost-effective public health intervention. However, an obvious requirement is that the fortified food(s) needs to be consumed in adequate amounts by a large proportion of the target individuals in a population. It is also necessary to have access to, and to use, fortificants that are well absorbed yet do not affect the sensory properties of foods. In most cases, it is preferable to use food vehicles that are centrally processed, and to have the support of the food industry (Hertrampf, 2002).

Fortification of food with micronutrients is a valid technology for reducing micronutrient malnutrition as part of a food-based approach when and where existing food supplies and limited access fail to provide adequate levels of the respective nutrients in the diet. In such cases, food fortification reinforces and supports ongoing nutrition improvement programs and should be regarded as part of a broader, integrated approach to prevent MNM, thereby complementing other approaches to improve micronutrient status (Verster, 2004).

In spite of implementing iron supplementation and nutrition education as well as public health measures through PHC system, Iron deficiency and its anemia is still one of the most common nutritional problems in Islamic republic of Iran. The extent and severity of anemia and iron deficiency, as well as health and economic consequences, highlight the need for prompt and efficient interventions. The food fortification program, which was a new approach, is being implemented in different countries and has become an integral part of the governments' overall economic development plan and has gained a key position in national health priorities.

In Iran, grains provide 63% of the total energy intake, and consumption of bread ranges from 230 to 505 gr/person/day (mean consumption of bread in Iran is 320 gr/person/day) (Sadighi et al, 2008). This dietary information indicates that wheat flour is an ideal vehicle for a fortification program in Iran.

3-1-3 Advantages and Limitations of Food Fortification

Food fortification offers a number of advantages but has also some limitations. These advantages and limitations are summarized as follows:

3-1-3-1 Advantages of Food Fortification

Being a food-based approach, food fortification offers a number of advantages over other interventions aimed at preventing and controlling micronutrient deficiency (World bank, 1994) (Allen et al, 2006). These include:

- If consumed on a regular and frequent basis, fortified foods will maintain body stores of nutrients more efficiently and more effectively than will intermittent supplements. Fortified foods are also better at lowering the risk of the multiple deficiencies that can result from seasonal deficits in the food supply or a poor quality diet.
- Fortification of widely distributed and widely consumed foods has the potential to improve the nutritional status of a large proportion of the population, both poor and wealthy.
- Fortification requires neither changes in existing food patterns – which are notoriously difficult to achieve, especially in the short-term – nor individual compliance.
- In most settings, the delivery system for fortified foods is already in place, generally through the private sector.
- In most cases, it is feasible to fortify foods with several micronutrients simultaneously.
- When properly regulated, fortification carries a minimal risk of chronic toxicity.
- Fortification is often more cost-effective than other strategies, especially if the technology already exists and if an appropriate food distribution system is in place.

3-1-3-2 limitations of food fortification

Although it is generally recognized that food fortification can have an enormous positive impact on public health, there are, however, some limitations to this strategy which are summarized as follows:

- A specific fortified foodstuff might not be consumed by all members of a target population. Conversely, everyone in the population is exposed to increased levels of micronutrients in food, irrespective of whether or not they will benefit from fortification.
- Infants and young children, who consume relatively small amounts of food, are less likely to be able to obtain their recommended intakes of all micronutrients from universally fortified staples or condiments alone; fortified complementary foods may be appropriate for these age groups. It is also likely that in many locations fortified foods will not supply adequate amounts of some micronutrients, such as iron for pregnant women, in which case supplements will still be needed to satisfy the requirements of selected population groups.
- Fortified foods often fail to reach the poorest segments of the general population who are at the greatest risk of micronutrient deficiency. This is because such groups often have restricted access to fortified foods due to low purchasing power and an underdeveloped distribution channel.
- While it is generally possible to add a mixture of vitamins and minerals to relatively inert and dry foods, such as cereals, interactions can occur between fortificant nutrients that adversely affect the organoleptic qualities of the food or the stability of the nutrients.

To ensure their success and sustainability, food fortification programs should be implemented in concert with poverty reduction programs and other agricultural, health, education and social intervention programs. Food fortification should thus be viewed as a complementary strategy for improving micronutrient status (Allen et al, 2006), (Verster, 1996).

3-1-4 Flour Fortification with Iron

Nutrient supplementation of foods was mentioned first in the year 400 B.C. by the Persian physician Melanpus, who suggested adding iron filings to wine to increase soldiers' potency. However, it was not a real supplementation and was more similar to fortification (Mejia, 1994).

Flour fortification with vitamin B1, niacin, and iron became possible in the USA in 1938. During the Second World War, flour fortification was introduced in the United Kingdom, too. There are now numerous countries in which flour producers are obliged by law to fortify their products with certain vitamins and minerals. Many other countries practice specific flour fortification on a voluntary basis (Allen et al, 2006).

3-1-4-1 Flour Fortification with Iron in Iran

In Iran there has been a formal nutrition strategy since 1994, and the reduction of iron deficiency anemia is part of it. Food fortification is one component of the nutrition strategy.

In 1996, flour fortification was conducted on a small scale in one of the districts of Isfahan (a province in the central of Iran) with 1200 population. The results showed an increase in hemoglobin in the target population. Based on the success of this activity and iron deficiency in Iran, The Iranian Ministry of Health and Medical Education launched a program for fortifying flour with iron on 31 May 2001 in Bushehr province, where the population was 800,000 (Sadighi et al, 2008). This province was selected due to a high prevalence of iron deficiency and anemia among women of child-bearing age (50% IDA according to a 1996 survey). Also, the province has an efficient health management system, a high level of commitment, as well as a strong primary health care network. Another conducive factor was the existing quality control labs for monitoring the amount of iron in the fortified flour. All the flour consumed was produced in two large-scale mills. Together they produced about 360 tons of fortified flour, which was sufficient for the daily needs of the population.

All requirements for the fortification process, equipment, quality assurance, and legislation have been met.

Flour is a good vehicle for fortification in Iran because:

- Wheat is a strategic product in Iran and all its purchasing, storage, processing, and distributing process is under governmental control.
- Bread is a strategic food for the Iranian population culturally and controlling its price, especially for low income households, is considered as one of the government's key strategies which is known as subsidy.
- According to National Household Food Consumption Survey, bread is a staple food and its consumption trend is predictable.
- International experiences suggest that iron as ferrous sulphate is stable and it does not change the color and taste of flour with the shelf life of less than two months.
- The main distributor of flour is the government.
- Iron fortification is feasible and economic for local flour industry.
- Flour fortification with multi-micronutrient is also possible and economical.

The advantages of flour such as long shelf life, excellent baking properties and easy digestibility ensures its success. Fortifying wheat flour with iron, make it possible to provide a special amount of additional iron to the diet of women, and indeed the entire population.

3-1-4-2 Evaluation of the Flour Fortification Program in Iran

In order to evaluate four fortification program in Iran three evaluations have been done in 2004, 2007, and 2009. The first study (conducted in 2004) was a field trial to evaluate program effectiveness. The intervention province (Bushehr) was compared with a control province (Fars) for anemia and iron-deficiency indicators. The target population was women aged 15-49 years and men aged 40-60 years. In this first evaluation, women in the intervention province (Bushehr) had a lower prevalence of low ferritin levels compared with women in the control province. Women from the two provinces did not differ in terms of hemoglobin levels or iron-deficiency anemia, and men from the two provinces did not differ significantly with regard to any of the tested parameters.

Importantly, although the flour fortification had no beneficial effect on anemia/iron deficiency in men, the iron fortification program also did not have the adverse effects on men, namely increased hemoglobin and ferritin levels. These findings suggested that the iron fortification program in Iran had a beneficial effect, but only on ferritin indicators among women.

This type of field trial study design could not be used for the second and third evaluations, as the flour fortification program had been expanded into other provinces (including Fars province) by the time the later studies were conducted. Therefore, the second and third evaluations were designed as before-and-after studies. The second evaluation (2007) showed that women and men in Bushehr province had a lower prevalence of low ferritin levels compared with the baseline (pre-intervention) sample.

In 2008, the World Bank suggested that parallel evaluations should be conducted in Bushehr and Golestan provinces. Therefore, another study was conducted in 2009, to evaluate the effectiveness and process of flour fortification with iron and folic acid in these two Iranian provinces. This study constitutes the third evaluation in Bushehr province and the first evaluation in Golestan province. This study showed that the program of wheat flour fortification with iron in Iran has had a beneficial effect on only the prevalence of iron deficiency in the studied women.

It is believed that some of the important factors diminish the beneficial effects of flour fortification on anemia in Iran include parasitic and infectious diseases, and other diseases prevalent in these areas (e.g. minor thalassemias). The relatively low availability of other micronutrients (e.g., vitamin A) should not be ignored.

In summary, we have shown that the flour fortification program in Iran is a useful component of a public health strategy aimed at improving iron deficiency status. However, in areas where anemia is not mainly due to iron deficiency, an iron fortification program might decrease the prevalence of iron deficiency without affecting the prevalence of anemia.

3-2 Iron and its Role in Human Health

3-2-1 Iron Absorption and Storage

Iron, by far the most abundant transition metal in the body, is an essential element for the utilization of oxygen, as well as a component of numerous oxidases and oxygenases. The major difficulty to overcome in iron assimilation by living cells is its bioavailability (Puntarulo, 2005). Soluble Fe (II) is readily oxidized in aqueous solutions to the essentially insoluble ferric hydroxide. In normal adults who ingest a diet free of iron supplements, the body content of 3-5 g is fairly well balanced among functional compounds, storage complexes, transport chelates, and ingestion and excretion.

Iron is transported and stored in specific proteins such as transferrin, lactoferrin, ferritin, and heme proteins. Serum transferrin is a carrier of iron in the blood that binds two Fe (III) ions with a high affinity. Under physiological conditions, only 30% of transferrin is saturated with iron (Pantopoulos and Hentze, 2000). Ferric iron is reduced to ferrous iron and delivered from the endosome into the cytosol. Excess iron is sequestered in ferritin, which is the main intracellular iron storage protein. Ferritin is composed of 24 subunits of H- and L-chains and can store up to 4500 atoms of Fe/molecule (Ponka et al, 1998).

The main part of body iron (60-70%) is utilized within hemoglobin in circulating red blood cells (Andrews, 2000). Other iron-reach organs are the liver and muscles. About 20-30% of body iron is stored in hepatocytes and in reticuloendothelial macrophages, to a large extent within ferritin and its degradation product hemosiderin. The remaining iron in body primarily exists in myoglobin, cytochromes, and iron containing enzymes. A healthy adult absorbs 1-2 mg of iron from the diet daily, which compensates for nonspecific iron losses by cell desquamation in the skin and the intestine. In addition, menstruating women physiologically lose iron through bleeding. Erythropoiesis requires approximately 30 mg iron/day, which is mainly provided through recycling iron via reticuloendothelial macrophages that ingest senescent red blood cells and release iron to circulating transferrin (Aisen et al, 2001). An average daily Iranian diet approximately contains 15 mg of iron (11.5 – 17.6),

from which less than 1-2 mg is absorbed (NNFTRI, 2005). In Iran, there is no data available on the bioavailability of nutrients, especially iron.

3-2-2 Biological Function of Iron

Despite the fact that iron is the second most abundant metal in the earth's crust , iron deficiency is the world's most common cause of anemia. When it comes to life, iron is more precious than gold. Iron absorption is the sole mechanism by which iron stores are physiologically manipulated. Iron is an essential nutrient for normal cellular functions and has the capacity to form a variety of coordination complexes with organic ligands in a dynamic and flexible mode (Papanikolaou and Pantopoulos, 2005). Iron exists in two oxidative states, ferrous, Fe (II), and ferric, Fe (III), which can donate or accept electrons. Although these redox reactions are important for biological reactions, they can also be hazardous to cells. This makes it a useful component of cytochromes, oxygen-binding molecules and some enzymes. However, excess of iron can potentially generate oxidative stress via increasing the production of reactive oxygen species (ROS). Redox reaction involving iron plays a key role in the formation of harmful free radicals and ROS that damage living cells via various pathways (Yamaji et al, 2004). Overproduction of ROS can damage different biomolecules and, consequently, can contribute to the initiation and development of several chronic pathologies such as diabetes, cancer, and cardiovascular diseases (Fraga and Oteiza, 2002) (Puntarulo, 2005).

3-2-3 Mechanism of Iron Toxicity

Iron is a critical element required for the normal functioning of all body cells, and is necessary for basic metabolic processes such as oxygen transport, DNA synthesis, cytochromes P450 enzyme oxidative metabolism and electron transport. Iron induces damage through nontransferrin iron in plasma.

One of the reasons iron is so ubiquitous in biology is its facility to redox cycle between the Fe2 and Fe3 state. In this process, it can generate free radicals, particularly in iron overload. The hydroxyl radical is the radical which has been most closely associated with iron-induced toxicity. There are probably other species which are important, such as the peroxynitrite (Puntarulo, 2005). Iron's

toxicity is largely based on Fenton and Haber-Weiss chemistry (Figure 1.), where catalytic amounts of iron are sufficient to yield hydroxyl radicals (OH°) from superoxide ($O_2^{\circ-}$) and hydrogen peroxide (H_2O_2), collectively known as ROS (Papanikolaou and Pantopoulos, 2005).

Figure 1. Fenton and Haber-Weiss reaction

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^- + OH \quad \textbf{(Fenton)}$$

$$Fe(III) + O_2^{\circ-} \longrightarrow Fe(II) + O_2$$

Net reaction:

$$H_2O_2 + O_2^{\circ-} \xrightarrow{Fe} OH^- + OH^{\circ} + O_2 \quad \textbf{(Haber-Weiss)}$$

As a matter of fact, ROS are inevitable byproducts of aerobic respiration and emerge as a result of incomplete reduction of dioxygen in mitochondria. ROS can also be generated during enzymatic reactions in other sub cellular compartments, such as in peroxisomes, endoplasmic reticulum, or the cytoplasm and are also produced by the membrane-bound NADPH oxidase complex, a multisubunit enzyme primarily expressed in phagocytic neutrophils and macrophages (Hampton et al, 1998). Finally, ferrous iron can also contribute as a reactant, rather than as a catalyst, to free radical generation through direct interaction with oxygen, via ferryl (Fe^{2+}-O) or perferryl (Fe^{2+}-O_2) iron intermediates (Figure. 2).

Figure 2. Direct interaction of iron with oxygen

$$Fe(II) + H_2O_2 \longrightarrow Fe(II)\text{-}O + H_2O$$

$$Fe(II) + O_2 \longrightarrow [Fe(II)\text{-}O_2 \longrightarrow Fe(III)\text{-}O_2^{\sigma-}] \longrightarrow Fe(III) + O_2^{\circ--}$$

It has been proposed that when $[O_2]/[H_2O_2] > 100$, these reactions may represent an important source for free radical generation in vivo (Huang, 2002).

In fact, free iron can cause cell damage by catalyzing the conversion of superoxide and hydrogen peroxide to free radical species that attack cellular membranes, proteins and DNA (Drog, 2002) (Fang et al, 2002). Free radicals are highly reactive species and may promote oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. An increase in the steady state levels of reactive oxygen (or nitrogen) species beyond the antioxidant capacity of the organism, called oxidative stress (or nitrosative stress), is encountered in many pathological conditions, such as chronic inflammation, ischemia-reperfusion injury, or neurodegeneration (Ischiropoulos and Beckman, 2003).

3-3 Oxidative Stress

3-3-1 Oxidative Stress Definition

Oxidative stress (OS) is an imbalance between free radicals and reactive oxygen species (ROS) and protective radical scavenging antioxidants (AOX) resulting from either an overproduction of ROS or a deficit in AOX protection (Terada 2006). Oxidative Stress (OS) is also used to describe the steady state level of oxidative damage in a cell, tissue, or organ, caused by the reactive oxygen species (ROS). This damage can affect a specific molecule or the entire organism. Reactive oxygen species, such as free radicals and peroxides, represent a class of molecules that are derived from the metabolism of oxygen and exist inherently in all aerobic organisms. Atmospheric oxygen in its ground-state is distinctive among the gaseous elements because it is a biradical, or in other words it has two unpaired electrons. This feature makes oxygen paramagnetic. It also makes oxygen very unlikely to participate in reactions with organic molecules unless it is "activated". The requirement for activation occurs because the two unpaired electrons in oxygen have parallel spins. According to Pauli's exclusion principle, this precludes reactions with a divalent reductant, unless this reductant also has two unpaired electrons with parallel spin opposite to that of the oxygen, which is a very rare occurrence. Hence, oxygen is usually non-reactive to organic molecules which have paired electrons with opposite spins. This spin restriction means that the most common mechanisms of

oxygen reduction in biochemical reactions are those involving transfer of only a single electron.

Activation of oxygen may occur by two different mechanisms: absorption of sufficient energy to reverse the spin on one of the unpaired electrons, or monovalent reduction. The biradical form of oxygen is in a triplet ground state because the electrons have parallel spins. If triplet oxygen absorbs sufficient energy to reverse the spin of one of its unpaired electrons, it will form the singlet state, in which the two electrons have opposite spins (Fig. 3). This activation overcomes the spin restriction and singlet oxygen can consequently participate in reactions involving the simultaneous transfer of two electrons (divalent reduction). Since paired electrons are common in organic molecules, singlet oxygen is much more reactive towards organic molecules than its triplet counterpart (Fang et al, 2002). The nomenclature of the various forms of oxygen is shown in Figure 3. The second mechanism of activation is by the stepwise monovalent reduction of oxygen to form superoxide (O^{-}_{2}), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and finally water according to the scheme shown in Figure 4. The first step in the reduction of oxygen forming superoxide is endothermic but subsequent reductions are exothermic (Gilbert, 2000).

Examples of oxygen free radicals are superoxide, hydroxyl, peroxyl, alkoxyl, and hydroperoxyl radicals.

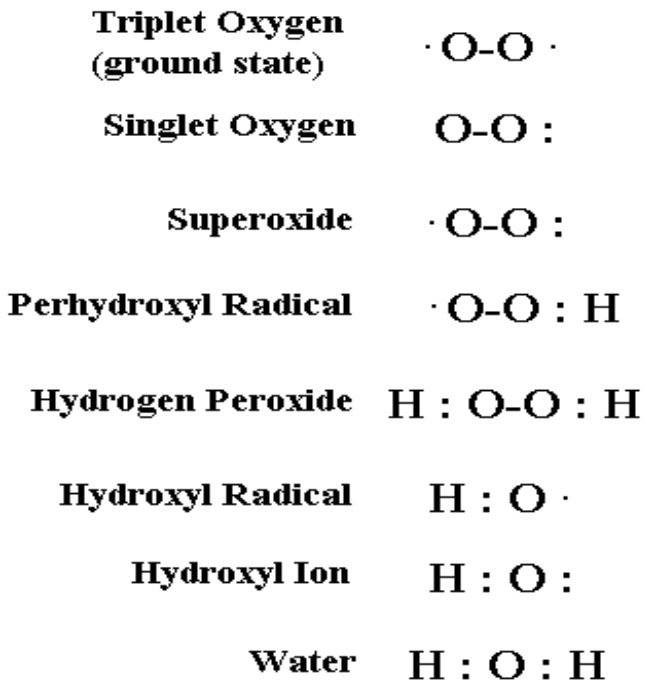

Figure 3: Nomenclature of the various forms of oxygen

The activation states of oxygen are shown in figure 4. The first step in the reduction of oxygen forming superoxide is endothermic but subsequent reductions are exothermic (Gilbert, 2000).

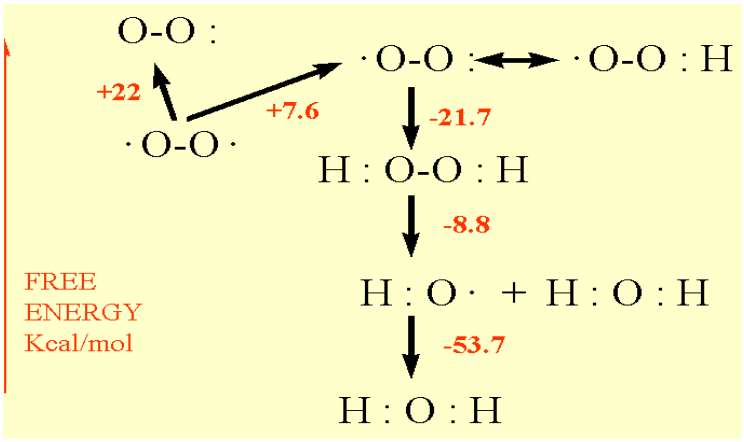

Figure 4: The activation states of oxygen

3-3-2 Oxidative Stress Sources

There are several sources by which the reactive oxygen species and free radical are generated. Most reactive oxygen species come from the endogenous sources as by-products of normal and essential metabolic reactions, such as energy generation from mitochondria or detoxification reactions involving the liver cytochromes P-450 enzyme system (Kurz et al, 2007). Exogenous sources include exposure to cigarette smoke, environmental pollutants such as emissions from automobiles and industries, consumption of alcohol in excess, asbestos, exposure to ionizing radiation, and bacterial, fungal or viral infections. The determinants of oxidative stress are regulated by an individual's unique hereditary factors, as well as his/her environment and characteristic lifestyle. Unfortunately, under the present day life-style conditions many people run an abnormally high level of oxidative stress that could increase the probability of an early decline in optimum body functions.

3-3-3 Oxidative Stress Measurement

Many methods for the measurement of OS have been proposed and several reviews have been written (Morrow et al, 1992), (Spiteller & Spiteller 1997), (Davies et al, 1999), (Pratico et al, 2004). However, there is no current consensus on the best method in terms of being the most useful, reliable, accurate or specific for different types of oxidative insults (Pryor 1999) (Collins et al, 2004).

In view of the lack of a "gold standard" method to assess free radical activity, three major approaches have been used: 1) determination of endogenous antioxidant levels; 2) measurement of the products of oxidized macromolecules; 3) direct detection of free radicals. For assessing endogenous antioxidant capacity, most studies have examined the concentrations of antioxidants (such as vitamin E, vitamin C, carotenoids, folate, GSH, and zinc) in plasma and cells and the cellular activities of antioxidant enzymes (such as glutathione reductase, SOD, catalase, and glutathione peroxidase) (Fang et al, 2002). Assessment of lipid peroxidation for evaluating oxidized macromolecules includes the analysis of lipid peroxides, isoprostanes, diene conjugates, and breakdown products of lipids. Among these products, Malondialdehyde is often

used as a reliable marker of lipid peroxidation. For assessing ROS-induced protein oxidation, most investigators have evaluated the production of protein carbonyls, loss of free thiol groups in proteins, and nitration of protein-bound tyrosine residues (Fang et al, 2002). Now a days, urinary excretion of 8-hydroxydeoxyguanosine may provide a useful, non-invasive means to assess whole-body DNA base oxidation in humans and animals (Jackson 1999).

There are three non invasive approaches to the detection of free radicals; the optical method, electron paramagnetic resonance (EPR), and nuclear magnetic resonance (NMR). The most sensitive of these approaches is the optical method in which emission light emits from the tissue, either intrinsically or extrinsically with lucigenin as an amplifier (Okuda et al, 1992).

3-3-4 Iron and Oxidative Stress

Perhaps the most dramatic discovery in recent years concerning mechanisms of oxidative damage in biological systems has been the finding that superoxide anions ($O_2^{\bullet -}$) can release iron from ferritin, providing free iron to catalyse the peroxidation of cell membranes (Keyer and Imlay, 1996). Therefore, $O_2^{\bullet -}$ causes an increase in the internal pool of free iron that may increase oxidative stress which, in turn, increases free iron concentration. Despite extensive literature on iron and lipid peroxidation, few studies have investigated the effects of iron fortified food consumption among non-anemic persons.

3-4 Animal Studies

The potential risks of an excess intake of iron in rats were first proposed over 50 years ago (Richmond, 1959). The hypothesis that high iron intake may be related to an increased risk of colorectal cancer has been developed on the basis of epidemiological evidence showing an association between high meat intake and cancer of the colon and rectum. Bobbs (Bobbs, 1990) proposed that the presence of iron in the colon would act as a catalyst for the production of free radicals by bacteria. Previous animal studies investigating the colonic response to high dietary iron intake have shown that iron may act as a tumor

promoter, whereas phytate has been shown to be protective, an effect presumed to be due to its iron-binding capacity. Although Soyars and Fischer (Soyars and Fischer, 1998) showed that iron had no effects on cell proliferation or the development of aberrant crypt foci (ACF) in rats, Davis and Feng (Davis and Feng, 1999) showed an increase in ACF with iron fortification at levels as low as 140 mg/kg. The difference in the results of the two above-mentioned studies may be due to the difference in the sampling site, use of different strains of rats or the period of fortification used.

The results of a study by Lund et al (Lund et al, 2001) provided evidence that excess dietary iron, taken over an extended period, might pose a significantly greater level of oxidative stress to the mucosal cells of the colon and particularly to those lining the cecum, equivalent to the proximal colon in rats. This is the site suggested by epidemiological studies to be most vulnerable to excess iron intake. Whole-animal studies show that intravenous administration of iron dextran in rats also induces oxidative stress (Lim and Vaziri, 2004).

In another animal study, the effects of parenterally administered 15 mg of iron dextran for 6 weeks on vascular oxidative stress were examined in mice. The results of this study showed that chronic parenteral administration of iron dextran markedly accelerated the thrombotic response to carotid artery injury, increased systemic and vascular ROS production, and impaired endothelium-dependent vasorelaxation in mice. Recent studies suggest that iron plays a key role in apoptosis induced by a variety of insults (Carlini et al, 2006) (Roth and Garrick, 2003). Xu in his study strongly suggested that the age-related iron accumulation in muscle contributed to increased oxidative damage and sarcopenia, and that calorie restriction effectively attenuates these negative effects (Xu et al, 2008). However, some other studies such as the Krik's study, found no difference in markers of oxidative damage between control and iron-loaded mouse livers (Krik et al, 2001). The relevance of these animal studies to vascular diseases and thrombosis in humans is unknown, but these data suggest that iron overload may increase oxidative stress and thrombotic risk (Day et al, 2003).

3-5 Human Studies

The effects of high dietary iron intake on oxidative stress have been particularly marked in women (Wurzelmann et al, 1996) (Kato, et al, 1999) (Isler et al, 2002) (Kurtoglu et al, 2003) (Amirkhizi et al, 2006), who are a more likely target group for iron supplementation. One study even showed that co-supplementation of ferrous salts with vitamin C exacerbates oxidative stress in the gastrointestinal tract (Fisher and Naughton, 2004). However, some studies have shown that iron supplementation is safe in anemic patients (Sareen et al, 2003) (Binkoski et al, 2004). One study suggested that iron and copper status might be associated with lipid peroxidation in subjects without metal overload (Arnaud et al, 2001).

Recent results have led to the proposal that superoxide plays an additional role in oxidative stress, which is increasing the level of intracellular free iron ions (Garner, 1997) (Henle and linn, 1997). Another human study concluded that oxidative damage was much higher in elderly males, suggesting a gender difference related to hormonal factors (Fano et al, 2001).

In one study, long-term alimentary iron overload resulted in a positive serum iron balance which, in turn, yielded an increased oxidative stress (Rehema et al, 1998).In one cross-sectional study, plasma iron levels were positively associated with lipid peroxidation (lasheras et al, 2003). Also another study indicated that in breast cancer patients, higher concentrations of serum iron were significantly correlated with higher levels of MDA (Bae et al, 2009).

A cohort study could not find any relationships between ferritin level and oxidative stress biomarkers (Senol et al, 2008). Furthermore, another study showed that ferritin played as a cytoprotective protein, minimizing oxygen free radical formation by sequestering intracellular iron (Orino et al, 2001).These results support other experimental studies (in vivo and in vitro) reporting that iron may store along the negatively changed lipid bilayer and promote lipid peroxidation and subsequently membrane damage (Leake and Rankin, 1990). However, a great part of the discrepancy may be due to the variability in the methods used, the population analyzed, study outcomes or types of epidemiological design. Results from a study on patients with acute myocardial

infarction showed an association between higher iron status and increased lipid peroxidation (Baykan et al, 2001).

Excessive intake of iron may predispose to mammary carcinogenesis due to the fact that free iron works as a catalyst for ROS generation. However, study results on the relationship between dietary iron intake and breast cancer are not consistent. Epidemiological studies have reported that there is no association between dietary intake and breast cancer risk (Adzersen et al, 2003) (Kabat et al, 2007). A large scale study conducted on Chinese female breast cancer patients reported that the intake of heme iron increased the risk of breast cancer (Kallianpur et al, 2008).

However with all the experimental evidence that ferrous iron promotes lipid peroxidation in vivo and in vitro (Heinecke et al, 1984) (Witztum, 1994) (Adzersen et al, 2003) (Kabat et al, 2007), epidemiological literature on this association in humans is scarce and inconsistent (Salonen, 1993) (Iribarren et al, 1998) (Bae et al, 2009). The same controversy exists regarding the role of iron stores in atherogenesis, and we have found an equal number of studies suggesting a detrimental role for iron (Salonen et al, 1993) (Tuomainen et al, 1998) (Liehr and Jones, 2001) (Kallianpur et al, 2008) as those suggesting a beneficial one (Sempos et al, 1994) (Orino et al, 2001) (Kirk et al, 2001).

3-6 Antioxidant Defense System in Human Body

Antioxidants are chemical compounds that inhibit oxidation (Halliwell, 2000). Some antioxidant defenses are as follows:

- The enzymes that catalytically remove free radicals and other reactive species such as SOD, CAT, and peroxidase. We can include "thiol-specific antioxidants" in this category as well.
- Proteins such as transferrin, haptoglobins, and metallothionein that minimize the availability of pro-oxidants such as iron and copper ions. Coeruloplasmin that oxidizes ferrous ions also fits in this category.

- Proteins, such as heat shock proteins protect biomolecules against damage (including oxidative damage) through other mechanisms.
- Low-molecular-mass agents scavenging ROS and RNS (including glutathione, ascorbic acid, α-tocopherol and uric acid) (Halliwell , 2000)

The composition of antioxidant defenses differs from tissue to tissue and between cell types within a tissue. Extracellular fluids have different protective mechanisms from the intracellular environments. According to the place of action, the components of the antioxidative defense system can be divided as follows:

Plasma antioxidant

- Water soluble: vitamin C, albumin, uric acid, and coeruloplasmin
- Lipid soluble: Vitamin E, carotenoids, and ubiquinol

Cellular antioxidant

- Water soluble: glutathione (GSH), vitamin C, SOD, CAT, GPx
- Lipid soluble: Vitamin E, carotenoids, and ubiquinol

Blood plasma albumin, urea, and bilirubin have proved to have substantial antioxidative properties (Mates et al, 2000).Blood plasma albumin, among its many other functions, serves as an important extra cellular antioxidant. Of extra cellular mainly blood plasma antioxidants, the role has been quite well described for bilirubin and urea. Urate binds iron and copper and scavenges hydroxyl radical, and peroxy radicals (Mates et al, 2000).

3-7 Overview

A lack of consistency among many studies on iron intake and oxidative stress is related to different parameters that are assessed in different studies and the fact that iron fortification is accepted to be an effective strategy to control anemia which has given way to forgetting or neglecting its adverse effects. These studies should be developed and integrated into plans for monitoring populations after the introduction of iron flour fortification program. There are very few studies on oxidative stress effects of iron intake from flour fortification programs among non-anemic persons.

4 METHODOLOGY

4-1 Study Design

This study had a special design and was implemented in two different parts: a double blind field trial and a before and after study.

Among 31 provinces of the Islamic Republic of Iran, Semnan province with a low prevalence of anaemia and iron deficiency was selected for this study. Then, two cities of this province were selected based on a number of criteria (each had to be equipped with a flour factory providing their flour needs, they had to neither import nor export flour, differences of geographical and socio-economic indicators between them had to be as little as possible, and no flour fortification program was launched in these two cities prior to the study).

Based on these qualifications, among the cities of this province, Damgan and Semnan were randomized as control and intervention cities, respectively. in the first part of the study that was a field trial with a control group, after baseline data collection, flour fortification started only in the intervention city (Semnan) and all kinds of bread in that city were fortified; so, in this part of the study, we had an intervention city with a control city (Damgan) and we followed all participants from these two cities for 32 weeks.

In the second part of the study, flour fortification started in Damgan as well. Since we could not keep this city as control in the second 32 weeks of the study, flour fortification was implemented both in Damgan and Semnan. This condition allowed us to follow fortification in Semnan for a longer period (64 weeks) as the second part of our study. It should be noted that the same participants who took part in the first phase or baseline data collection also participated in the second and third phases and they were allowed to maintain their regular food habits. An overview of the study design is presented in Figure 5.

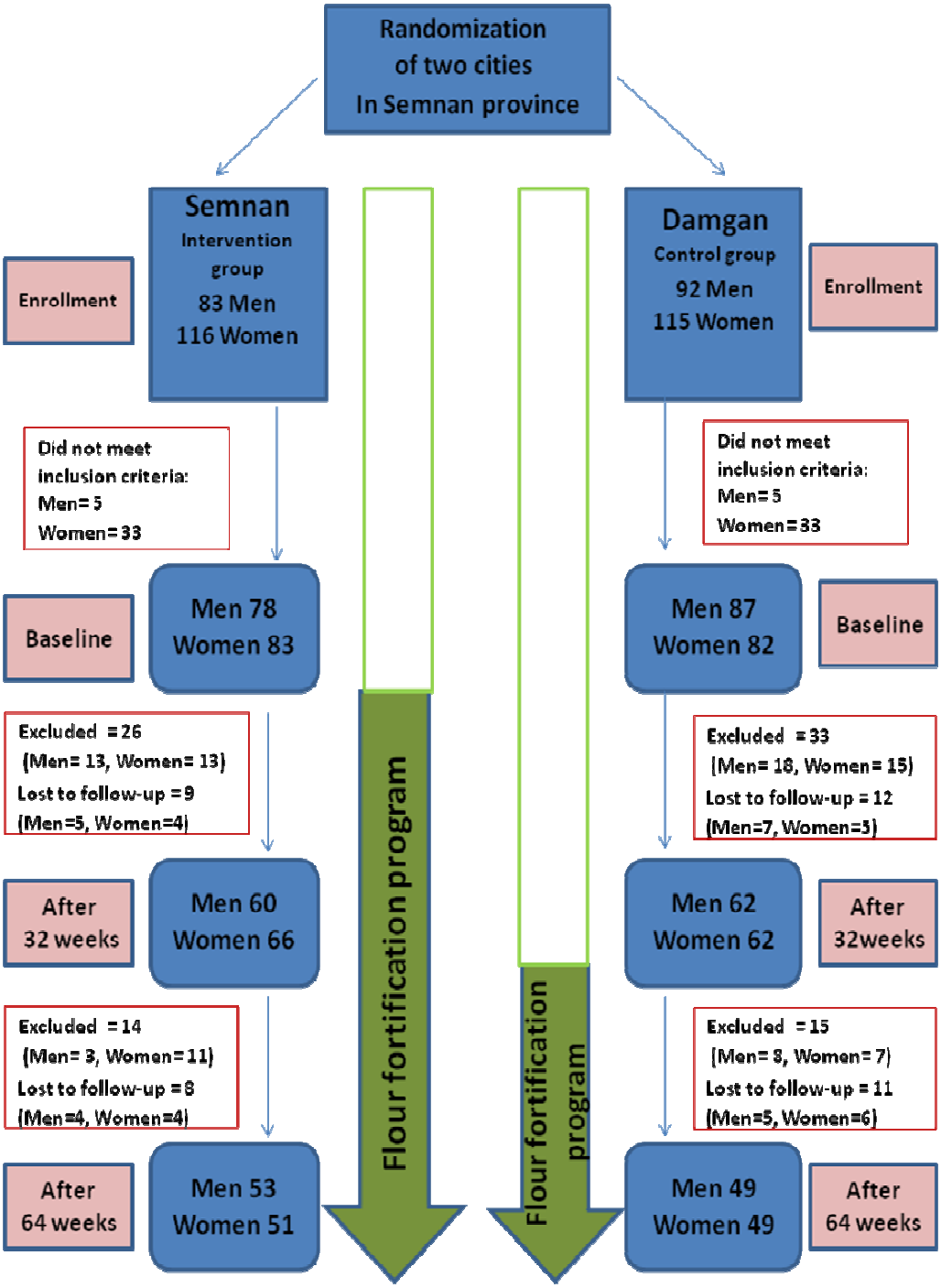

Figure 5. An overview of study design

4-2 Sample Size

Based on the results of a related survey (Amirkhizi et al., 2006) and the following equation below for two sided alternative:

$$N = \frac{[Z(1-\alpha/2) + Z(1-\beta)]^2 (SD_1^2 + SD_2^2)}{(\mu_1 - \mu_2)^2} = 34$$

$\beta = 0.2$ $\mu_1 - \mu_2 = 0.3$ $SD_1 = 0.5$ $SD_2 = 0.4$ $\alpha = 0.05$

The sample size needed for this study was 34 in each group and a total of 102 volunteers for all.

But based on the prevalence of Iron deficiency in this province, data gathering for three times, and possible cases of loss to follow-up, we selected 100 persons in each group at baseline data collection. The samples were accessed based on a multi-stage stratified method by calling up their houses in a cluster manner. So, we had a total number of 400 volunteers in the first phase.

4-3 Sampling Criteria

Volunteers were recruited from among 40- to 65-year-old, non- anemic and apparently healthy men and women who lived in Semnan and Damgan (Semnan province) in 2007**.** The inclusion and exclusion criteria were as follows:

Inclusion criteria:

- Completing the letter of consent
- Being apparently healthy
- Being 40-65 years of age
- Not following special diets for the last 2 months before the study
- Not having any major organ system diseases
- Not consuming any Iron or multivitamin supplements at least two months before the study
- Not being pregnant and lactating

Exclusion criteria

- Smoking
- High blood pressure (systolic B.P. more than 140 mmHg or diastolic B.P. more than 90 mmHg)
- Anemia (Hb≤12mg/dl for women, and Hb≤13mg/dl for men)
- Moving out of their city for more than three months
- Having undergone any kind surgical operation
- Using iron or multivitamin supplements
- Death

Volunteers were selected if their complete blood count (CBC) was within the normal range and met the criteria of entering the study. The purpose and the nature of the study and what was expected from the subjects in terms of participation were explained to them and each volunteer signed a written consent prior to entering the study. The protocol of the study was approved by the Ethical Committee of National Nutrition and Food Technology Research Institute (NNFTRI), and Ministry of Health and Medical Education, Tehran, Iran. It should be noted that in the first phase of the study, those who suffered anaemia were referred to a physician for treatment. Volunteers could withdraw their participation at any time during the study.

4-4 Data Collection

Volunteers were asked to be present at the Health Care Center of the city in each phase of data collection. Based on our study design, we had three phases for data collection; at the beginning of the study (the first phase), at the end of the 32^{nd} week (the second phase), and at the end of the 64^{th} weeks (the third phase). Examination and interviewing (completing the information forms) were the methods of data collection. In each of the three phases, demographic, anthropometric, blood pressure, information on food intake (3-day 24-hour recall and a food frequency questionnaire with 103 items), physical activities (a short version of IPAQ), and some oxidative stress biomarkers were the main data that were collected (a copy of the study forms is available in the appendix section).

4-5 Anthropometric Measurements

At all three phases of the study, anthropometric measurements of the volunteers were taken. Body weight was measured with 100 gr accuracy, using digital scales (Seca) with minimum clothing and without shoes. Height was measured with a non-stretchable tape (Seca) with 0.1 cm accuracy. Body mass index (BMI) was calculated using the equation; BMI=weight (kg)/height (m) ^{2} and was recorded on a special information form.

4-6 Dietary Assessment

At all three phases of the study, a 3-days 24-hour recall and a Food Frequency Questionnaire with about 103 items (quantitative), which was validated by the Centre for Endocrinology and Metabolism Research of Shahead Beheshti University of Medical Sciences, Tehran, Iran (Fazeltabar et al, 2006) were taken from each volunteer by a trained nutritionist. Quantities were estimated from photographs of portion sizes and from household measures (Ghafarpour et al, 2007). Volunteers were allowed to maintain their regular food habits. Data was translated into energy and nutrients (energy, micro and macronutrients) using Food Dorosti Processor (FDP) II software, which has been modified for Iranian foods.

4-7 Laboratory measurements

8-12 ml blood samples were drawn from the volunteers in the morning after 12-14 hours of fasting at the three phases (baseline, after 32 weeks, and after 64 weeks). Blood was taken from the median cubital vein and divided in two parts, either with or without anticoagulant, in acid washed tubes. About 1.5-2 ml was used to measure hemoglobin and cell blood count (CBC) in standard tubes containing anticoagulant. Sera from clot samples were recovered after 1 hour at room temperature (RT) followed by centrifugation at 2500g at RT for 20 minutes. In order to avoid repeated serum defrosting, we divided all our lab tests in six parts, so serum samples were divided in six parts and stored at -80°C awaiting further analysis.

The serum samples were analyzed for:

Dependent variables	Methods
Hemoglobin	Cell counter
Ferritin	immunoturibidimetric
Serum Iron	Ferene (colorimetric)
TIBC	Immunoturibidimetric [Transferrin mg/dl X 1.25]
Transferrin	immunoturibidimetric
STfR	Elisa
MDA	Spectrophotometric
TAC	Colorimetric
SOD	Elisa
GPx	Elisa
Oxidized-LDL	Elisa
PC	Elisa
Albumin	Bromocresol green
Uric acid	Colorimetric (Toos)
Total protein	Biuret
Total Bilirubin	Colorimetric (DCA)
α-Tochopherol	HPLC
β- Carotene	HPLC
Lycopene	HPLC
Retinol	HPLC

Hemoglobin:

Hemoglobin determinations were performed by the Orphee MYTHEC 18 clinical analyzer from a tube of well-mixed EDTA-anticoagulated blood filled to a predetermined level. In this assay, all forms of hemoglobins are converted to the colored protein cyanomethemoglobin and measured by a colorimeter. The coefficient of variation of this method was 3%.

Ferritin:

The method for the measurement of ferritin was immuno-turbidimetry using Parsazmun's kits (Parsazmun Co. INC) on the Hitachi 912 clinical analyzer. Latex-bound Ferritin antibodies react with the antigen in the sample to form an antigen/antibody complex. Following agglutination, this is measured turbidimetrically. Turbidity is proportional to the ferritin concentration, and is measured at 700 nm (primary wavelength).The intra-assay coefficient of variation for this method using this kit was 3.8% and the inter-assay coefficient of variation was 4.7%.

Serum iron:

Serum iron was measured using Ferene method (Burtis and Ashwood, 1994). Parsazmun's iron assay kit (Parsazmun Co. INC) provides a simple convenient means of measuring Ferrous and/or Ferric ion in samples. In the assay, ferric carrier protein dissociates ferric into solution in the presence of an acid buffer. After reduction to the ferrous form (Fe^{2+}), iron reacts with Ferene S to produce a stable colored complex and give absorbance at 593 nm. A specific chelate chemical is included in the buffer to block copper ion (Cu^{2+}) interference. The coefficient of variation of this method was 2.93%.

Transferrin (and TIBC):

In order to measure transferring saturation (TS), transferrin was measured using Parsazmun's kits (Parsazmun Co. INC) (immunoturibidimetric method) (Cook et al, 1974). In this method, a reaction occurs between transferrin and polyclonal antibodies that yields a turbid solution. The turbidity of the solution is proportional to the concentration of transferrin in the sample. In order to measure TIBC (µg/dl), this concentration [transferrin (mg/gl)] multiplied by 1.25. The coefficient of variation of this method was 3%.

Serum transferrin receptor:

Serum transferrin receptor was measured using DRG's kit (DRG diagnostic, Germany). This assay is based on the microplate sandwich enzyme immunoassay technique using two different monoclonal antibodies specific for sTfR. Samples or standards are pipetted into wells of a microplate pre-coated

with a monoclonal antibody that can capture sTfR, thereby immobilizing sTfR to the well. After washing away any unbound protein, a second anti-sTfR monoclonal antibody conjugated to horseradish peroxidase is added. The conjugated antibody completes the sandwich. After washing away unbound conjugated antibody, the amount of conjugate remaining in the well is proportional to the amount of sTfR initially captured. The amount of conjugated enzyme in the well is measured by incubation with a chromogenic substrate. The intra-assay coefficient of variation for this method was 7.1% and the inter-assay coefficient of variation was 6.4%.

Malondialdehyde (MDA):

Serum MDA levels were measured as an index of lipid peroxidation according to the Thiobarbituric acid (TBA) spectrophotometric method that was introduced by Satoh (Satoh, 1978) with minor modifications. The coefficient of variation of this method was 5%.

Total Antioxidant Capacity (TAC):

2, 2'-azinobis (3-ethylbenzothiazolin 6-sulfate) or ABTS produces ABTA cation radical ($ABTS^{\circ+}$) under the influence of potassium persulfate. The original ABTS solution is colorless turning to green-blue after adding potassium persulfate and forming cation radical. This solution becomes stable after 24hrs and keeps its stability for another 48 hours. Maximum absorption occurs at 415 nm, 645nm, 734nm, and 815nm. Adding antioxidant solution to this in a given time, color intensity decrease depending on antioxidant activity and concentration. Therefore, decolorization is expressed as a percent of $ABTS^{\circ+}$ inhibition based on the difference of the primary and secondary absorbance divided by primary absorbance multiplied by 100. Standard curve of bovine serum albumin (BSA) is used to convert inhibition percentage to g/dl. The intra-assay coefficient of variation for this method was 1.6% and the inter-assay was 4%.

Superoxide dismutase (SOD):

The SOD activity of serum was measured with Cayman's Superoxide Dismutase assay kit (Cayman chemical company, USA). This method utilizes a tetrazolium salt for detecting superoxide radicals generated by xanthine oxidase

and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. Scheme of the super oxide dismutase assay is shown in figure 6.

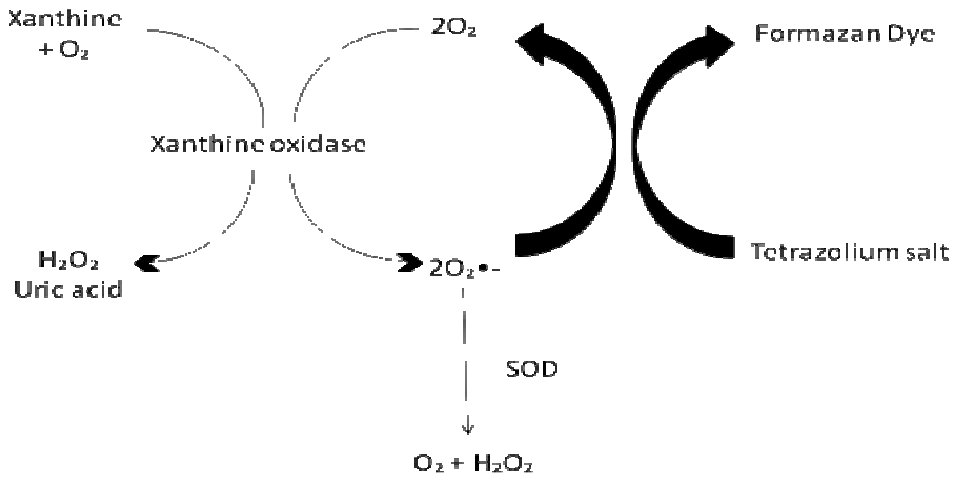

Figure 6. Scheme of the superoxide dismutase assay

The intra-assay coefficient of variation for this method was 3.2% and the inter-assay coefficient of variation was 3.7%.

Glutathione peroxidase (GPx):

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect cells from oxidative damage.

Serum GPx activity was evaluated with Cayman GPx assay kit (Cayman chemical company, USA). Cayman's GPx assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR) (Pagila and Valentine, 1967). Oxidized glutathione (GSSG), produced upon the reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. This reaction is presented in figure 7.

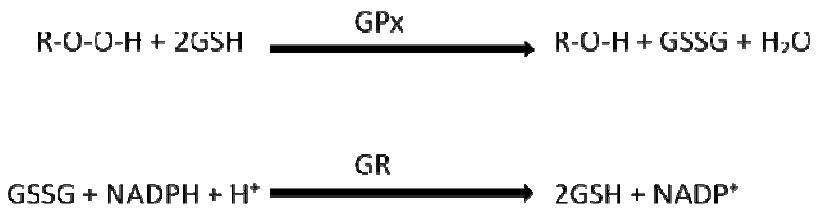

Figure 7. Scheme of glutathione peroxidase assay

The intra-assay coefficient of variation by using this method is 5.7% and the inter-assay coefficient of variation was 7.2%.

Oxidized LDL:

The Mercodia Oxidized LDL ELISA kit (Sweden) was used for quantitative measurement of oxidized low density lipoprotein. Mercodia Oxidized LDL ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the oxidized apolipoprotein B molecule. During incubation, oxidized LDL in the sample reacts with anti-oxidized LDL antibodies bound to microtitration well. After washing, which removes non-reactive plasma components, a peroxidase conjugated anti-human apolipoprotein B antibody recognizes the oxidized LDL bound to the solid phase. After a second incubation and a simple washing step that removes unbound enzyme labeled antibody, the bound conjugate is detected by reaction with 3, 3', 5, 5'-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint, and is then read spectrophotometrically. Serum oxidized LDL levels were measured by a competitive ELISA utilizing a specific murine monoclonal antibody mAb-4E6 (Holveot et al, 2001).
The coefficient of variation of this method using this kit was 8.3%.

Protein Carbonyl (PC):

Protein carbonyls were measured by using OxiSelect Protein Carbonyl ELISA kit (Cell Biolabs, USA) following the protocol provided by the manufacturer. Cell Biolabs' Protein Carbonyl ELISA Kit is an enzyme immunoassay developed for rapid detection and quantitation of protein carbonyls. The quantity of protein carbonyls in protein samples is determined by comparing its absorbance with that of a known reduced/oxidized BSA standard curve. BSA standards or protein samples (10µg/ml) are adsorbed onto a 96-well plate for 2 hrs at 37ºC. The protein carbonyls present in the sample or standard are derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. The protein carbonyl content in the unknown sample is determined by comparing with a standard curve that is prepared from predetermined reduced and oxidized BSA standards (Buss et al, 1985). In terms

of assay variation, based on this test, the intra-assay coefficient of variation was 6% and the inter-assay coefficient of variation was 8%.

Serum Albumin:

Serum albumin levels were measured using Bromocresol green method (Parsazmun Co. INC). In this method, Albumin in the sample reacts with bromocresol green in an acid medium forming a coloured complex that can be measured by spectrophotometry (Doumas et al, 1971). The coefficient of variation for this method was 1.79%.

Uric Acid:

Uric acid is a metabolic product of exogenous or endogenous purine bases. Since uric acid is found in human serum in relatively low concentrations, it is necessary to use specific and sensitive methods for its determination. In this study, uric acid levels were measured using colorimetric (TOOS) method (Jelikic-Stankov et al, 2003). This is a specific enzymatic method. It involves the catalytic oxidation of uric acid with the enzyme uricase to allantoin with the formation of hydrogen peroxide. The peroxide, the concentration of which is directly proportional to the concentration of uric acid, can be determined by colorimetric methods using N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS). Intra-assay coefficient of variation for this method was 2.2% and inter-assay coefficient of variation was 2.7%.

Total Protein:

The serum total protein, as its name implies, represents the sum total of numerous different proteins, many of which vary independently of each other. Total protein was measured using Biuret method Parsazmun kit (Reinhold, 1953). Proteins form a purple colored complex with cupric ions in an alkaline solution. The reaction takes its name from the simple compound Biuret which reacts in the same way. The intensity of the purple color is measured at 540 nm / yellow green filter and compared with a standard serum of known protein concentration. The coefficient of variation for this method was 1%.

Total Bilirubin:

Total bilirubin was measured using the colorimetric (DCA) method (Rand and Dipasqua, 1962). At least four distinct bilirubin species exist in the serum: Direct-reacting bilirubin (DB) consisting of mono and diconjugated bilirubin (β and γ-Bilirubin), δ-fraction which is bilirubin tightly bound to albumin, and unconjugated α-bilirubin which is water soluble and bound to albumin. The reaction between DB and diazotised dichloroaniline leads to a compound, azubilirubin, whose absorbance is directly proportional to the concentration of DB in the specimen and is measured at 550 nm. The coefficient of variation for this method was 2%.

Serum α–Tocopherol, β-Carotene, Lycopene, and Retinol:

Serum levels of beta-carotene, lycopene, α-tocopherol, and retinol were determined using the HPLC method as described by Neyestani et al (Neyestani et al, 2007) but with some minor modifications. Serum samples obtained from patients were immediately frozen and stored at -80°C until they were used for HPLC analysis. After examining various chromatographic conditions, the following method was found to be the most reliable. After protein precipitation with ethanol, carotenoids, α-tocopherol, and retinol were extracted using n-hexane, which was then evaporated under nitrogen flow at 40°C. The sediment was reconstituted with a mixture of mobile phase (methanol: acetonitrile: tetrahydrofurane, 50:45:5, v/v/v) and diethyl ether (2:1, v/v), 20μL of which was injected to the column Novopack C18.

Instrument specification for HPLC:

Column	C18 Novapak 3.9 ×150
Flow rate	1.5 ml/min
Temperature	ambient room
Pressure	1100 PSA
Wavelent	retinol: 325, α-tocopherol: 292nm, lycopene: 472nm, β-carotene: 450nm
Retention time	retinol: 1.5 min, retinol acetate: 1.9 min, α-tocopherol: 5.6, lycopene: 12.5, beta carotene: 12.9

Run time	20 min
Loop volume	20µl

The intra-assay coefficient of variation for this method was 6.5% and the inter-assay coefficient of variation was 4.8%.

4-8 Quality Control & Quality Assurance

Once flour fortification starts, the flour industry must have in place a strong quality assurance/quality control (QA/QC) system, and appropriate food control and regulatory monitoring procedures must be followed to ensure that safe and adequately fortified flour (according to national standards) is continuously produced to meet the dietary and nutritional needs of the population (Pena-Rosas et al, 2008). In our study, we organized a workshop to train laboratory managers of the flour factories. They were trained to take at least 2-3 fortified flour samples every day. Using the spot test (Nalubola and Nestel, 2000), they were asked to check the fortification process in the factory (as QC) .Central Food and Nutrition lab (CFNL) in the province also had regular visits from flour factories and took some samples of fortified flour for quantitative test to detect the exact amount of iron in the fortified flour (As QA). In CFNL, the iron content was assayed using a spectrophotometric method (AACC 40-41B). This method determines iron content by reaction with orthophenanthroline and spectrophotometric measurement (Nalubola and Nestel, 2000). In terms of the iron level in bread (or flour), we expected to have four levels of fortification: low was defined as 25–39.9 mg/kg, good as 40–65.9 mg/kg, acceptable as 66–79.9 mg/kg and high as ≥80 mg/kg. These ranges were taken from the guidelines of the iron assay method (Allen et al, 2006). To ensure that the observations were true and due to flour fortification, we also took some regular fortified flour samples from bakeries and sent them to a different laboratory to check the exact amount of iron and compared these results with the results of CFNL as double check.

4-9 Statistical analysis

Data was expressed as means and standard deviations.When the distribution of variables was not normal, the data were expressed in median and inter-quartile range (IQR). The normality of the distribution of variables was tested using the kolmogorov smirnov test. The Naperian logarithm transformation of values was performed to make data fit to the normal distribution when the distribution was not normal. The analysis of variance (ANOVA) was used to identify any difference among groups in the three phases. Multiple comparisons among phases were done using the Bonferroni and Dunnett post-hoc tests. Nonparametric test of Kruskal-Wallis was used for those distributions which were not normal. Linear relationships between serum values were examined using Pearson's correlation coefficient.

Analysis of the data was done by SPSS software version 14 using the dependent test in each group and the independent test between groups regarding the type and the distribution of variables. Analysis of consumed foods was done by Food Processor II software. For all hypotheses, a significance level of $P<0.05$ was considered statistically significant.

4-10 Research variables:

Variables	Independent Variable	Dependent Variable	Quantitative		Qualitative		Measurement tools	Unit
			Continues	Discrete	Nominal	Ordinal		
Survey group	*				*		_	Control and intervention group
Time of intervention	*		*				weeks after fortification	0, 32, and 64 weeks
Age	*			*			Identification Card	year
Sex	*				*		See person	Men-women
Weight	*		*				Using Seca scale	kg
Height	*		*				Using Seca height measurements	cm
BMI	*		*				Calculator	Kg/m^2
BMI	*					*	-	25> = 0 25-29.9 = 1 >30 = 2
Literacy level	*					*	Question	1-No literacy 2- Primary school 3-Junior and high school 4-Diploma/ university
Iron supplements Intake	*				*		Question	Yes- no
Surgery history	*				*		Question	Yes-no
Blood donation history	*				*		Question	Yes-no
Inflammation history in two weeks before	*				*		Question	Yes-no
Alcohol intake	*				*		Question	Yes-no
Hemoglobin		*	*				CBC	gr/dl
Hematocrit		*	*				CBC	%
Serum iron		*	*				Lab test	µgr/dl
Total Iron Binding Capacity (TIBC)		*	*				Lab test	%
Transferrin saturation(TS)		*	*				Lab test	%
Serum ferritin(Sf)		*	*				Lab test	ng/ml
Serum Transferrin Receptor(STfR)		*	*				Lab test	nmol/l
Total Protein		*	*				Lab test	gr/dl
Albumin		*	*				Lab test	gr/dl
Uric acid		*	*				Lab test	mg/dl

Research variables Continue….

Variables	Independent Variable	Dependent Variable	Quantitative		Qualitative		Measurement tools	Unit
			Continues	Discrete	Nominal	Ordinal		
Bilirubin		*	*				Lab test	mg/dl
Blood pressure		*	*				Lab test	mmHg
Malondialdehyde (MDA)		*	*				Lab test	µmol/l
Total Antioxidant Capacity(TAC)		*	*				Lab test	µmol/l
Super Oxide Dismutase(SOD)		*	*				Lab test	U/ml
Glutathione Reductase(GPx)		*	*				Lab test	U/ml
Protein Carbonyl(PC)		*	*				Lab test	nmol/mg
Oxidized LDL(OxLDL)		*	*				Lab test	µmol/l
α -Tocopherol		*	*				Lab test	mgr/dl
β-Carotene		*	*				Lab test	µgr/dl
Lycopene		*	*				Lab test	µgr/dl
Retinol		*	*				Lab test	µgr/dl
Iron intake	*		*				Quantitative FFQ	mg/day
Copper Intake	*		*				Quantitative FFQ	mg/day
Zinc Intake	*		*				Quantitative FFQ	mg/day
Vitamin C, Intake	*		*				Quantitative FFQ	mg/day
Vitamin A, Intake	*		*				Quantitative FFQ	IU/day
Vitamin E, Intake	*		*				Quantitative FFQ	mg/dl
Multivitamin Supplement intake	*		*				Question	Yes-no
Physical activity Level	*					*	Questionnaire	-Under active -Moderate -Active
Cigarette smoking	*			*			Questionnaire	Number/day
Energy intake	*		*				24 hour recall	Kcal
Protein intake	*		*				24 hour recall	gr/day
Fat intake	*		*				24 hour recall	gr/day
PUFA intake	*		*				24 hour recall	mg/day

5 RESULTS

Based on our study design, data was presented in three sections:

5-1 Baseline characteristics before intervention

5-2 Field trial after 32 weeks

5-3 Effects of iron fortified flour consumption after 64 weeks (before and after study)

5-1 Baseline Characteristics before Intervention

To investigate any difference among the study groups in the two cities at the beginning of the study, the main study variables were compared in this part.

Of the 396 volunteers who entered the study at baseline data collection, full data was obtained from 330 volunteers due to inclusion and exclusion criteria (Table 5-1-1).

Table 5-1-1. The number of males and females at the time of baseline data collection in the two cities

Gender	City		Total
	Damgan	Semnan	
Male	87	78	165
Female	82	83	165
Total	169	161	330

The inverse relationship between oxidative stress and education is to be expected because educated persons are less likely to be obese (Winkleby et al, 1998) and are more likely to adopt dietary and other lifestyle practices consistent with higher antioxidant status.

As Table 5-1-2 and 5-1-3 show, there was no significant difference in the level of literacy among study groups in two cities at the time of baseline data collection.

Table 5-1-2. Level of literacy among males at the time of baseline date collection in the two cities

city	Illiterate	Primary school	Junior and High school	Diploma and university	P value
Damgan (n=87)	1%	25%	24%	50%	0.59
Semnan (n=78)	4%	21%	20%	55%	

The Fisher's exact test showed no significant difference in the two groups

Table 5-1-3. Level of literacy among females at the time of baseline date collection in two cities

city	Illiterate	Primary school	Junior and High school	Diploma and university	P value
Damgan (n= 82)	9%	42%	14%	35%	0.68
Semnan (n= 83)	13%	33%	18%	36%	

The Chi-square test showed no significant difference in the two groups

As mentioned earlier in statistical analysis, the normality of the distribution of variables was tested using the Kolmogorov-Smirnov test. To compare the mean of the variables among study groups between two cities at baseline, independent t-test was used.

The means and standard deviations (SD) of age, BMI, systolic and diastolic blood pressure are presented in table 5-1-4.

Aging is a process of deleterious and progressive changes in multiple organ systems. With aging, oxidant production in skeletal muscle may exceed the antioxidant capacity to buffer oxidants, resulting in oxidative damage. The mean age of males and females, as shown in table 5-1-4, was not significant at baseline in the two cities.

Body mass index was measured by dividing weight in kilograms by height in meters squared. The normal range for BMI in this study was between 20 and 25. A BMI more than 25 and less than 30 was considered overweight, and a

BMI more than 30 was considered obese. Means and SDs of BMI of the subjects did not show any significant difference among males and females in two cities at the baseline of the study (table 5-1-4).

Hypertension is associated with increased vascular oxidative stress; however, there is still debate whether oxidative stress is a cause or a result of hypertension. Based on the data presented in table 5-1-4, there was no significant difference in systolic and diastolic blood pressure among study groups at the baseline of the study.

Table 5-1-4. Baseline characteristics of males and females in intervention group (Semnan) and control group (Damgan)

Variables	Sex	City		
		Damgan Mean(± SD)	Semnan Mean(± SD)	P value
Age (year)	Male*	50.9 ± 6.9	50.4 ± 6.4	0.87
	Female**	49.4 ± 6.5	50.0 ± 6.0	0.68
BMI (kg/m^2)	Male	26.9 ± 5.2	26.8 ± 3.6	0.97
	Female	30.2 ± 4.2	31.3 ± 5.7	0.20
Systolic blood pressure (mmHg)	Male	119.9 ± 13.2	120.7 ± 12.1	0.73
	Female	117.9 ± 15.8	121.7 ± 16.3	0.17
Diastolic blood pressure (mmHg)	Male	75.3 ± 8.4	78.3 ± 11.1	0.21
	Female	78.5 ± 10.6	82.1 ± 12.0	0.15

Independent t-test showed no significant difference between the two groups in the two cities

*n= 78 in Semnan, n=87 in Damgan
**n=83 in Semnan, n= 82 in Damgan

The means and SDs of iron status and some endogenous antioxidant parameters in males and females at the baseline of the study are presented in table 5-1-5.

To determine the iron status in the body, hemoglobin, ferritin, serum iron, total iron binding capacity, transferrin saturation, and serum transferrin receptors

were measured in our study. Measuring the level of serum iron is one of the most frequently performed diagnostic tests for trace elements. Evaluation of iron (IRN), total iron binding capacity (TIBC), and the calculated ratio of iron concentration and total iron binding capacity [(IRN/TIBC) * 100 =% TS] are useful for the differential diagnosis of anemia and iron overload and monitoring the treatment of certain disease conditions. Serum ferritin is believed to be the best determinant for evaluating body iron stores as 1ng of serum ferritin corresponds to 8 mg of stored iron in the absence of inflammation, MI, or other critical events. The soluble fraction of transferrin receptor can be also measured which is considered to be the best parameter describing iron stores even in the presence of inflammation.

Mean values of the hemoglobin and ferritin showed no significant difference in males and females at baseline in the two cities (table 5-1-5).

Independent t-test did not show significant changes in serum transferrin receptor, serum iron, TIBC, and transferrin saturation (TS) among males and females in the two cities at the baseline of the study (table 5-1-5).

Table 5-1-5. Mean and standard deviation of iron status and some endogenous antioxidant parameters of males and females at baseline in Semnan and Damgan

Variables	Sex	City		
		Damgan Mean(± SD)	Semnan Mean(± SD)	P value
Hemoglobin (gr/dl)	Male*	14.3 ± 0.8	14.3 ± 0.8	0.98
	Female**	12.8 ± 0.7	13.0 ± 0.6	0.56
Ferritin (ngr/ml)	Male	143.5 ± 47.1	157.5 ± 57.9	0.09
	Female	98.9 ± 39.7	98.4 ± 40.2	0.65
Serum transferrin receptor (µgr/ml)	Male	1.2 ± 0.2	1.3 ± 0.3	0.13
	Female	1.3 ± 0.4	1.4 ± 0.6	0.13
Serum iron (µgr/dl)	Male	101.2 ± 29.6	100.1 ± 29.7	0.97
	Female	90.2 ± 31.1	94.0 ± 31.4	0.50
Total iron binding capacity (TIBC)(µgr/dl)	Male	321.2 ± 75.9	299.3 ± 72.6	0.15
	Female	295.2 ± 72.9	289.9 ± 76.1	0.70
Transferrin saturation (TS) (%)	Male	33.9 ± 11.9	31.7 ± 11.5	0.24
	Female	30.6 ± 12.6	28.5 ± 11.9	0.33
Albumin (gr/dl)	Male	3.9 ± 0.2	4.0 ± 0.3	0.13
	Female	3.9 ± 0.3	4.0 ± 0.3	0.08
Total Bilirubin (µgr/d)	Male	1.11 ± 0.55	1.03 ± 0.42	0.60
	Female	0.89 ± 0.35	0.82 ± 0.28	0.26
Total protein (gr/dl)	Male	7.0 ± 0.5	7.1 ± 0.4	0.16
	Female	7.0 ± 0.4	7.2 ± 0.6	0.11
Uric acid (mg/dl)	Male	4.9 ± 1.2	5.3 ± 1.0	0.13
	Female	4.6 ± 1.1	4.6 ± 1.1	0.80

Independent t-test did not show any significant differences between two groups in two cities

*n= 78 in Semnan, n=87 in Damgan
**n=83 in Semnan, n= 82 in Damgan

Serum levels of albumin, total bilirubin, total protein, and uric acid are shown in the table 5-1-5 as well.

Uric acid is the final product of purine metabolism in humans. The final two reactions of its production catalyzing the conversion of hypoxanthine to xanthine and the latter to uric acid are catalyzed by the enzyme xanthine oxidoreductase,

which may attain two inter-convertible forms, namely xanthine dehydrogenize or xanthine oxidase. The latter uses molecular oxygen as electron acceptor and generates superoxide anion and other reactive oxygen products. The role of uric acid in conditions associated with oxidative stress is not entirely clear. Evidence mainly based on epidemiological studies suggests that increased serum levels of uric acid are a risk factor for cardiovascular diseases where oxidative stress plays an important pathophysiological role.

As early as 1959, it was suggested that bilirubin might be an antioxidant. Bilirubin can act as an important cytoprotector of tissues that are poorly equipped with antioxidant defense systems including myocardium and nervous tissue. Bilirubin, when bound to human albumin and at concentrations present in normal human plasma, protects albumin-bound linoleic acid from peroxyl radical-induced oxidation in vitro (Baranano et al, 2002).

Albumin, the major serum protein, has a cystein residue in position 34 which is not involved in a disulfide bond and may exist in different oxidation states: as a fully reduced sulfhydryl group, as a mixed disulfide with cystein, glutathione or homocystein, or in higher oxidation states such as sulfenic, sulfinic or sulfonic acid. Serum albumin is known to act as an antioxidant. The oxidation state of human serum albumin is discussed as a marker for systemic oxidative stress and it has been shown that disulfide content of serum albumin is increased by strenuous exercise in older people and in the course of different diseases.

Comparing mean values for albumin, total bilirubin, total protein, and uric acid, as shown in table 5-1-5, showed no significant difference between males and females in two cities at the baseline of the study.

Mean values of some oxidative stress biomarkers and dietary antioxidants are presented in table 5-1-6.

Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation. Lipid peroxides are formed as a consequence of free radical attack on polyunsaturated fatty acids. The polyunsaturated fatty acids break down in the presence of iron or other metals forming aldehydes e.g.

malondialdehyde (MDA) via the formation of cyclic peroxides and endoperoxides (precursors of prostaglandin).
To determine the antioxidant capacity of the serum, we used total antioxidant capacity (TAC). Antioxidants play an important role in preventing the formation of and scavenging free radicals and other potentially toxic oxidizing species.

Oxidized LDL is a form of low-density lipoprotein (LDL), or "bad" cholesterol that has been bombarded with oxygen to yield free radicals when it enters into the wall of an artery.

Superoxide dismutases (SOD) are a class of enzymes that catalyse the dismutation of superoxide into oxygen and hydrogen peroxide. They are an important antioxidant defense in nearly all cells exposed to oxygen.

Glutathione peroxidase (GPx) is the general name of an enzyme family with peroxidase activity whose main biological role is to protect the organism from oxidative damage. One of the biochemical functions of glutathione peroxidase is to reduce free hydrogen peroxide to water.

Protein carbonyl (PC) is the most general indicator and by far the most commonly used marker of protein oxidation. Redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to the cation binding locations on proteins and with the aid of further attack by H_2O_2 or O_2 can transform side-chain amine groups on several amino acids (such as Lysine, Argenine, Proline) into carbonyls.

The independent t-test did not show significant changes in MDA, TAC, SOD, GPx, PC, and oxidized LDL of males and females in the two cities at the baseline of the study.

Lycopene and β-carotene are two strong antioxidant carotenoids. Carotenoids contribute to antioxidant activity in cellular lipids, support normal cellular growth and renewal, and assist healthy levels of immune cell activity. Carotenoids promote cardiovascular health including support for a healthy antioxidant response to LDL cholesterol. Beta carotene is one of the most important natural antioxidants. There is no RDA for beta carotene.

Alpha tocopherol is the main form of vitamin E and is most commonly studied dietary antioxidant supplement in clinical trials.

Retinol, the main circulating form of vitamin A, has a free radical scavenging potential.

The mean values of dietary antioxidants such as serum retinol (vitamin A), alpha tocopherol (Vitamin E), beta carotene, and lycopene, as presented in table 5-1-6, showed no significant difference between males and females in two cities at the baseline of the study.

Table 5-1-6. Mean and standard deviation of oxidative stress and dietary antioxidants of female and male participants at the baseline and after 32 weeks in Semnan and Damgan

Variables	Sex	City		
		Damgan Mean(± SD)	Semnan Mean(± SD)	P value
Malondialdehyde, MDA (µmol/L)	**Male***	**3.0 ± 0.9**	**2.9 ± 1.1**	0.92
	Female**	**2.9 ± 1.1**	**2.8 ± 1.0**	0.52
Total antioxidant capacity, TAC ((µmol/L)	**Male**	**1.82 ± 0.21**	**1.87 ± 0.24**	0.87
	Female	**1.86 ± 0.18**	**1.83 ± 0.25**	0.48
Superoxide dismutase, SOD (U/ml)	**Male**	**0.59 ± 0.20**	**0.58 ± 0.20**	0.30
	Female	**0.55 ± 0.25**	**0.58 ± 0.23**	0.38
Glutathione peroxidase, GPx (U/ml/min)	**Male**	**178.2 ± 39.2**	**174.6 ± 35.3**	0.39
	Female	**181.4 ± 31.8**	**183.5 ± 35.5**	0.95
Protein carbonyl, PC (nmol/mg)	**Male**	**2.22 ± 0.26**	**2.29 ± 0.27**	0.20
	Female	**2.25 ± 0.54**	**2.26 ± 0.59**	0.69
Oxidized LDL (µmol/L)	**Male**	**185.1 ± 64.2**	**181.8 ± 61.6**	0.75
	Female	**198.4 ± 61.5**	**188.9 ± 81.0**	0.68
Vitamin A (µgr/dl)	**Male**	**78.6 ± 22.6**	**73.3 ± 26.1**	0.23
	Female	**73.1 ± 24.0**	**73.2 ± 24.4**	0.92
Vitamin E (mg/dl)	**Male**	**1.39 ± 0.40**	**1.33 ± 0.50**	0.34
	Female	**1.52 ± 0.56**	**1.49 ± 0.52**	0.68
Lycopene (µgr/dl)	**Male**	**57.5 ± 21.8**	**50.0 ± 21.0**	0.06
	Female	**64.3 ± 26.4**	**53.1 ± 19.5**	0.13
β-carotene (µgr/dl)	**Male**	**27.9 ± 14.1**	**25.0 ± 12.1**	0.44
	Female	**26.5 ± 12.7**	**26.8 ± 13.3**	0.26

Independent T-test showed no significant difference between the two groups in the two cities

*n= 78 in Semnan, n=87 in Damgan

**n=83 in Semnan, n= 82 in Damgan

In order to find differences in subjects' dietary intakes among study groups at the baseline of the study, data obtained from three-day 24-hour recall for intake of total energy, total protein, total fat, and polyunsaturated fatty acids (PUFA)

and food frequency questionnaire (quantitative) for vitamin C, vitamin A, vitamin E, iron, zinc, and copper were studied.

Comparing means and standard deviations of energy, macronutrients, iron, zinc, copper, and antioxidant vitamins intake as presented in table 5-1-7 did not show any significant differences between males and females in two cities at the baseline of the study.

The World Health Organization and many national health agencies have independently conducted studies and even though they differ slightly, all have concluded that our daily protein requirement should be between 10 to 15 percent of our daily caloric intake. In the present study, the mean value of the percent of protein from energy was about 12.5 percent for both males and females and there was no significant difference at the baseline of the study in each gender in two cities.

USDA Dietary Guidelines of 2005 recommends that total fat intake should count between 20 to 35 percent of energy for adults. No more than 10 percent of energy should be from saturated fats (there is no dietary guideline for Iranian population at the moment). In our study, the means of energy intake are presented in table 5-1-7. The mean percentage of energy produced from fat in males and females was between 25 to 26 percent and about 29 percent at the baseline, respectively. There were no statistically significant differences within males and females in fat intake at the baseline of the study (table 5-1-7).

The National Heart, Lung, and Blood Institute recommends that up to 10% of total daily energy should be from PUFA. Double bonds in PUFA can lead to formation of free-radicals and reactions with oxygen to form unstable lipid peroxide compounds containing the same unstable oxygen bond found in hydrogen peroxide. Although polyunsaturated fatty acids may have a protective effect against atherosclerosis, moderation in consumption is recommended. In our study, the mean value of the percent of PUFA from energy in males and females was between 8.3 and 8.9 percent and between 10.3 and 10.7 percent at baseline, respectively. Females' PUFA intake was a little bit more than what is recommended (10%) but there was no significant difference between females in the two cities (table 5-1-7).

Vitamin C is the major water-soluble antioxidant and its reducing power is used in radical and nonradical redox reactions. It is considered to be the most important antioxidant in extra cellular fluids and has many cellular activities of an antioxidant nature as well. It was shown that ascorbic acid was far more effective in inhibiting lipid peroxidation initiated by a peroxyl radical initiator than other plasma components, such as protein thiols, urate, bilirubin, and α-tocopherol. The recommended amount by RDA for non-smoker adult males and females is 90 and 75 mg, respectively. In this study, the mean value of vitamin C intake for males was about 90 mg but females had a higher vitamin C intake than RDA recommendation (93.3 mg in Semnan and 89.5 mg in Damgan). Although the mean value of vitamin C in females was more than RDA (75 mg), there was no significant difference in the two cities at the baseline of the study (table 5-1-7).

Vitamin A participates in several primordial functions in human systems playing a role in visual acuity, cellular proliferation and differentiation. It also shows antioxidant properties and immunological activities. The recommended amount by RDA for Vitamin A is 900 mg / day RAE. As table 5-1-7 shows, the mean value of vitamin A intake for males and females in the two cities was less than RDA, but there was no statistically significant difference in males and females in two cities at the baseline of the study.

Vitamin E is a fat-soluble vitamin which has antioxidant properties. Natural vitamin E exists in eight chemical forms (alpha-, beta-, gamma-, and delta-tocopherol and alpha-, beta-, gamma-, and delta-tocotrienol) that have varying levels of biological activity. Alpha- (or α-) tocopherol is the only form that is recognized to meet human requirements. Recommended dietary allowance for vitamin E (α-tocopherol) for adult males and females is 15 mg. In the present study, the mean values of vitamin E intake for males and females in Semnan and Damgan were 9.3, 9.1, 9.2 and 8.7 mg, respectively. There were no statistically significant differences in vitamin E intake in males and females at the baseline of the study (table 5-1-7).

Iron, the most prevalent metal in the body, reacts with hydrogen peroxide and catalyzes the generation of highly reactive hydroxyl radicals, thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton

and Haber–Weiss reaction. Excessive iron intake from either foods or dietary supplements can be a source of ROS, although results from epidemiologic studies are inconsistent. The oxidative stress produced by dietary intake of iron might be modified by endogenous oxidant and antioxidant capabilities that may act in concert to provide a coordinated network of protection against ROS accumulation and oxidative damage. The current iron RDA for postmenopausal women (ages 50+) and all men is 8 mg per day. The RDA for premenopausal women (ages 19 to 50) is 18 mg per day.
The difference in mean intake of iron, as shown in table 5-1-7, was not significant in males and females in Semnan and Damgan at the baseline of the study.

The relevance of zinc for proper functioning of the entire immune system is already well documented. It is quite clear that antioxidants and micronutrients in the diet, such as zinc, influence the development and function of immune cells, the activity of stress-related proteins and antioxidant enzymes and help to maintain genomic integrity and stability. Recommended dietary allowance for zinc for adult males and females is 11 and 8 mg, respectively. In the present study, the mean values of zinc intake in Semnan and Damgan males and females were 7.75, 8.34, 6.85 and 7.26 mg, respectively. There was no statistically significant difference between males and females in zinc intake at the baseline of the study (table 5-1-7).
Copper (Cu), a redox active metal, is an essential nutrient for all species studied to date. It is an integral part of many important enzymes involved in a number of vital biological processes. Although normally bound to proteins, Cu may be released and become free to catalyze the formation of highly reactive hydroxyl radicals. Data obtained from in vitro and cell culture studies are largely supportive of Cu's capacity to initiate oxidative damage and interfere with important cellular events. Vitamin E is generally protective against Cu-induced oxidative damage. Interestingly, a deficiency in dietary Cu also increases cellular susceptibility to oxidative damage. Recommended dietary intake of copper (Cu) for adults is 900 µgr/day. In the present study, the mean values of copper intake for males and females in Semnan and Damgan were 1.89, 2.02, 1.67 and 1.81 mg, respectively. There were no statistically significant

differences in copper intake in males and females at the baseline of the study (table 5-1-7).

Table 5-1-7 Energy, macronutrients, iron, zinc, copper, and antioxidant vitamins intake of males and females at baseline in the intervention group (Semnan) and the control group (Damgan)

Variables	Sex	City		
		Damgan Mean(± SD)	Semnan Mean(± SD)	P value
Energy (kcal)	Man*	2174.8 ± 501.5	2218.9 ± 646.0	0.68
	Women**	1569.7 ± 501.2	1687.2 ± 471.6	0.08
Protein (gr)	Male	66.3 ± 17.5	68.8 ± 22.1	0.53
	Female	48.8 ± 17.4	53.2 ± 17.1	0.17
Total fat (gr)	Male	60.2 ± 25.1	65.2 ± 29.6	0.33
	Female	50.7 ± 17.7	54.1 ± 14.1	0.46
Poly unsaturated fatty acid (PUFA)(gr)	Male	20.1 ± 4.1	21.9 ± 4.6	0.63
	Female	18.8 ± 3.8	19.3 ± 4.2	0.54
Vitamin C (mg)	Male	88.8 ± 24.1	91.2 ± 28.5	0.78
	Female	93.3 ± 32.3	89.5 ± 26.2	0.55
Vitamin A(µg)	Male	676.6 ± 274.9	709.4 ± 230.1	0.17
	Female	725.9 ± 210.8	755.2 ± 264.1	0.38
Vitamin E (mg)	Male	9.1 ± 3.7	9.3 ± 4.0	0.46
	Female	8.7 ± 3.0	9.2 ± 2.2	0.15
Iron intake (mg)	Male	13.7 ± 4.1	12.9 ± 3.4	0.26
	Female	12.1 ± 4.0	11.0 ± 2.9	0.11
Zn intake (mg)	Male	8.34 ± 2.1	7.75 ± 1.7	0.33
	Female	7.26 ± 2.1	6.85 ± 1.8	0.26
Cu intake (mg)	Male	2.02 ± 0.6	1.89 ± 0.4	0.14
	Female	1.81 ± 0.6	1.67 ± 0.4	0.09

Independent T-test showed no significant difference between the two groups in the two cities

*n= 78 in Semnan, n=87 in Damgan
**n=83 in Semnan, n= 82 in Damgan

Based on the results of this part of the study, there was no significant difference in iron status and oxidative stress biomarkers at baseline between the study groups.

5-2 Field trial

Effects of iron fortified flour consumption on iron status and oxidative stress in the intervention group and its comparison with the control group after 32 weeks

In this part, based on our study design, flour fortification program started only in Semnan (intervention city) in its two flour factories after baseline data collection. So, the volunteers in this city consumed bread fortified with 30 mg/kg iron but in Damgan (control city), usual flour was distributed without any extra iron. We followed the volunteers in these two cities for 32 weeks. After 32 weeks, the second part of data collection started. The normality of the distribution of the variables in the second phase of the study was tested using Kolmogorov-Smirnov test. To compare the mean of variables between the study groups in the two cities at baseline and after 32 weeks, paired t-test was applied.

The means and standard deviations (SDs) of BMI, systolic, and diastolic blood pressure at baseline and after 32 weeks are presented in table 5-2-1 and table 5-2-2.

Table 5-2-1 Mean and standard deviation of BMI, systolic, and diastolic blood pressure of male participants from Semnan and Damgan at the beginning and after 32 weeks

Variables	City	Males (n=60 in Semnan and n=62 in Damgan)		
		Baseline Mean(± SD)	After 32 weeks Mean(± SD)	P value
BMI (kg/m^2)	Damgan	26.8 ± 3.6	26.8 ± 3.7	0.96
	Semnan	26.9 ± 5.2	26.8 ± 5.6	0.61
Systolic blood pressure (mmHg)	Damgan	120.7 ± 12.1	119.0 ± 14.0	0.28
	Semnan	119.9 ± 13.2	121.0 ± 11.7	0.40
Diastolic blood pressure (mmHg)	Damgan	78.3 ± 11.1	78.3 ± 11.3	0.96
	Semnan	75.3 ± 8.4	75.2 ± 13.5	0.95

Paired T-test did not show any significant differences among males in the two cities

The idea that obesity (BMI ≥ 30 kg/m^2) is a state of chronic oxidative stress and inflammation, even in the absence of other CVD risk factors, shows the importance of developing effective preventions and treatment strategies for obesity. While Keaney and colleagues (Keaney et al, 2003) suggest that obesity is independently associated with oxidative stress, the close association between obesity and other conditions that potentially increase oxidative stress leaves open the possibility of residual confounding factors; for example, the association between oxidative stress and obesity may be related to other unmeasured variables.

Table 5-2-2 Mean and standard deviation of BMI, systolic, and diastolic blood pressure of female participants from Semnan and Damgan at the beginning and after 32 weeks

Variables	City	Females (n=66 in Semnan and n=62 in Damgan)		
		Baseline Mean(± SD)	After 32 weeks Mean(± SD)	P value
BMI (kg/m^2)	Damgan	31.3 ± 5.7	31.5 ± 6.1	0.13
	Semnan	30.2 ± 4.2	30.1 ± 4.1	0.60
Systolic blood pressure (mmHg)	Damgan	121.7 ± 16.3	119.8 ± 15.6	0.16
	Semnan	119.7 ± 15.7	118.1 ± 16.9	0.36
Diastolic blood pressure (mmHg)	Damgan	82.1 ± 12.0	79.1 ± 10.7	0.26
	Semnan	78.5 ± 10.6	76.0 ± 11.0	0.38

Paired T-test did not show any significant differences among females in the two cities

Mean values of BMI, systolic, and diastolic blood pressure of males and females in the two cities were constant throughout this part of the study. There were no statistically significant differences in BMI, systolic, and diastolic blood pressure between the study groups in the two cities at the beginning of the study nor they significantly changed after 32 weeks.

According to literature review, physical activity is one of the main factors that induces oxidative stress. Sudden vigorous exercise has been shown to increase oxidative stress because increased aerobic metabolism is a source of oxidative stress, whereas long-term moderate exercise may upregulate antioxidative enzymes and decrease indices of oxidative stress. In order to find differences in physical activity, we used the short format of self-administrated International Physical Activity Questionnaire (IPAQ). The purpose of the IPAQ is to provide a

set of well-developed instruments that can be used internationally to obtain comparable estimates of physical activity. Based on this questionnaire, three levels of physical activity are proposed: low, moderate, and high. However, to summarize data, we just mentioned the percentage of low active and high active individuals in our tables. Based on the data on Table 5-2-3 and Table 5-2-4, there were no significant changes between the study groups at the baseline and after 32 weeks.

Table 5-2-3 Physical activity of male participants from the two cities at the beginning and after 32 weeks

city	low active %		High active %		P value
	Baseline	After 32 weeks	Baseline	After 32 weeks	
Damgan (n= 62)	57.4	55.1	10.6	10.2	0.9
Semnan (n= 60)	56.0	54.2	12.0	12.5	0.9

The Chi-square test showed no significant difference between the two groups

The percentage of low active males in Damgan and Semnan at baseline was 57.4% and 56%, respectively. After 32 weeks, however, it decreased a little in Damgan and Semnan to 55.1% and 54.2% respectively, but there was no statistically significant difference between them (table 5-2-3). The percentage of high active men in Damgan and Semnan at baseline was 10.6% and 12%, respectively. Although this rate in Damgan and Semnan changed to 10.2% and 12.5% respectively, there was no significant difference between them (table 5-2-3).

The percentage of low active women in Damgan and Semnan at baseline was 70.8% and 70.6% respectively which increased to 71.4% and 72.2% after 32 weeks, respectively; however, the difference was not significant (table 5-2-4). The percentage of high active women in Damgan and Semnan at baseline was 6.3% and 7.8% which decreased to 6.1% and 7.4% after 32 weeks, respectively. The difference, however, was not significant (table 5-2-4).

Table 5-2-4 Physical activity of female participants from the two cities at the beginning and after 32 weeks

city	Low active %		High active %		P value
	Baseline	After 32 weeks	Baseline	After 32 weeks	
Damgan (n= 62)	70.8	71.4	6.3	6.1	0.8
Semnan (n= 66)	70.6	72.2	7.8	7.4	0.7

The Fisher's exact test showed no significant difference between the two groups

After 32 weeks of fortified flour consumption in Semnan, in order to investigate any significant difference in iron status, we compared the mean and SD of hemoglobin, ferritin, serum iron, TIBC, TS, and serum transferrin receptors of males and females with baseline values. These data are presented in Table 5-2-5 and Table 5-2-6. However, the paired t-test did not show significant differences.

The mean value of the parameters related to iron status such as hemoglobin, ferritin, serum receptor transferrin, serum iron, TIBC, and transferrin saturation in Damgan females worsened after 32 weeks, as presented in table 5-2-5, but the difference was not significant. Meanwhile in Semnan, where all subjects had consumed fortified bread for 32 weeks, these parameters remained constant or rather improved, but there was no significant difference in their parameters, as well (table5-2-5). It should be noted that not all subjects in our study were anemic.

Table 5-2-6 shows mean and standard deviation of iron status in male participants at baseline and after 32 weeks. Based on the data presented in this table, the iron status of men in Semnan is a little better than Damgan but paired t-test showed no statistically significant differences.

Table 5-2-5 Mean and standard deviation of iron status and some endogenous antioxidant parameters in female participants from Semnan and Damgan at baseline and after 32 weeks

Variables	City	Females (n=66 in Semnan and n=62 in Damgan)		
		Baseline Mean(± SD)	After 32 weeks Mean(± SD)	P value
Hemoglobin (gr/dl)	Damgan	13.0 ± 0.5	12.9 ± 0.9	0.39
	Semnan	12.8 ± 0.7	12.8 ± 0.8	0.97
Ferritin (ngr/ml)	Damgan	98.4 ± 40.2	96.8 ± 37.5	0.41
	Semnan	98.9 ± 39.7	100.8 ± 39.3	0.82
Serum transferrin receptor (µgr/ml)	Damgan	1.4 ± 0.6	1.5 ± 0.6	0.12
	Semnan	1.3 ± 0.4	1.3 ± 0.3	0.23
Serum Iron (µgr/dl)	Damgan	94.0 ± 31.4	86.8 ± 26.5	0.10
	Semnan	90.2 ± 31.1	94.9 ± 30.0	0.32
Total Iron Binding Capacity (TIBC) (µgr/dl)	Damgan	289.9 ± 76.1	297.8 ± 84.0	0.30
	Semnan	295.2 ± 72.9	289.8 ± 82.0	0.35
Transferrin Saturation (TS) (%)	Damgan	31.9 ± 13.6	28.1 ± 7.6	0.18
	Semnan	28.9 ± 12.6	29.0 ± 13.4	0.78
Albumin (gr/dl)	Damgan	4.0 ± 0.3	3.9 ± 0.3	0.08
	Semnan	3.9 ± 0.3	4.1 ± 0.6	0.05
Total Bilirubin (µgr/d)	Damgan	0.82 ± 0.28	0.73 ± 0.21	0.02
	Semnan	0.89 ± 0.35	0.90 ± 0.32	0.86
Total protein (gr/dl)	Damgan	7.2 ± 0.6	7.1 ± 0.4	0.12
	Semnan	7.0 ± 0.4	7.3 ± 0.5	0.00
Uric acid (mg/dl)	Damgan	4.6 ± 1.1	4.0 ± 0.9	0.00
	Semnan	4.6 ± 1.1	4.4 ± 1.0	0.16

In Tables 5-2-5 and 5-2-6, some serum antioxidant parameters such as albumin, uric acid, total protein, and total bilirubin are also presented. Serum albumin (3.9 to 4.1 gr/dl) and total protein (7.0 to 7.3 gr/dl) significantly increased in females from Semnan ($p<0.05$) but in females from Damgan, uric acid (4.6 to 4mg/dl) and total bilirubin (0.82 to 0.73µgr/dl) significantly decreased ($p<0.05$). Data also suggested that among males in Semnan, albumin (3.9 to 4.1gr/dl) and total protein (7.0 to 7.4gr/dl) significantly increased ($p<0.05$), but total bilirubin (1.11 to 0.89µgr/dl) significantly decreased ($p<0.05$). Meanwhile in Damgan, uric acid (5.3 to 4.9mg/dl) and total bilirubin (1.03 to 0.89µgr/dl) significantly decreased in males ($p<0.05$).

Table 5-2-6 Mean and standard deviation of iron status and some endogenous antioxidant parameters in male participants from Semnan and Damgan at baseline and after 32 weeks

Variables	City	Males (n=60 in Semnan and n=62 in Damgan)		
		Baseline Mean(± SD)	After 32 weeks Mean(± SD)	P value
Hemoglobin (gr/dl)	Damgan	14.3 ± 0.8	14.3 ± 1.1	0.79
	Semnan	14.3 ± 0.8	14.4 ± 0.8	0.40
Ferritin (ngr/ml)	Damgan	157.5 ± 57.9	158.1 ± 57.1	0.67
	Semnan	143.5 ± 47.1	145.5 ± 45.1	0.70
Serum transferrin receptor (µgr/ml)	Damgan	1.3 ± 0.3	1.4 ± 0.3	0.20
	Semnan	1.2 ± 0.2	1.2 ± 0.2	0.65
Serum Iron (µgr/dl)	Damgan	100.1 ± 29.7	100.0 ± 32.2	0.86
	Semnan	101.2 ± 29.6	101.6 ± 31.5	0.95
Total Iron Binding Capacity (TIBC) (µgr/dl)	Damgan	299.3 ± 72.6	314.4 ± 73.3	0.62
	Semnan	321.2 ± 75.9	316.5 ± 66.3	0.71
Transferrin Saturation (TS) (%)	Damgan	34.9 ± 11.7	32.0 ± 14.4	0.35
	Semnan	31.2 ± 11.3	29.3 ± 11.2	0.36
Albumin (gr/dl)	Damgan	4.0 ± 0.3	4.0 ± 0.2	0.85
	Semnan	3.9 ± 0.2	4.1 ± 0.5	0.03
Total Bilirubin (µgr/d)	Damgan	1.03 ± 0.42	0.89 ± 0.29	0.004
	Semnan	1.11 ± 0.55	0.89 ± 0.25	0.007
Total protein (gr/dl)	Damgan	7.1 ± 0.4	7.1 ± 0.4	0.48
	Semnan	7.0 ± 0.5	7.4 ± 0.6	0.00
Uric acid (mg/dl)	Damgan	5.3 ± 1.0	4.9 ± 0.8	0.00
	Semnan	4.9 ± 1.1	5.1 ± 1.2	0.18

In order to compare mean and SD of oxidative stress parameters of the male and female participants after 32 weeks of fortified flour consumption in Semnan with Damgan with baseline values, we used MDA, TAC, SOD, GPx, protein carbonyl, and Ox-LDL.

Malondialdehyde (MDA) mean and standard deviation of male and female participants are illustrated in table 5-2-7 and table 5-2-8. Although the mean value of MDA increased in participants from Semnan (male and female), where fortified bread was consumed, paired t-test showed no significant difference in this parameter in males and females from Semnan and Damgan after 32 weeks.

Mean and standard deviation of participants for TAC at baseline and after 32 weeks are presented in table 5-2-7 and table 5-2-8. To identify any change throughout the study (32 weeks), the paired t-test was used. Although participants from Semnan (male and female) experienced a much more

decrease in TAC compared to participants from Damgan, these changes were not significant after 32 weeks.

Mean and standard deviation of SOD and GPx at baseline and after 32 weeks are shown in table 5-2-7 and table 5-2-8. SOD and GPX are important enzymes that protect body against oxidative stress. Although mean values of SOD and GPx after 32 weeks of fortified bread consumption increased in participants from Semnan compared to the values of the participants from Damgan, paired t-test showed no statistically significant difference in males and females after 32 weeks in the two cities.

Table 5-2-7 Comparing means and standard deviations of oxidative stress and dietary antioxidants among females at the baseline and after 32 weeks in Semnan and Damgan

Variables	City	Females (n=66 in Semnan and n=62 in Damgan)		
		Baseline Mean(±SD)	After 32 weeks Mean(±SD)	P value
Malondialdehyde MDA (µmol/L)	Damgan	**2.8 ± 1.0**	**2.7 ± 0.8**	**0.46**
	Semnan	**2.9 ± 1.1**	**3.0 ± 0.9**	**0.48**
Total antioxidant capacity TAC (µmol/L)	Damgan	**1.83 ± 0.25**	**1.79 ± .10**	**0.64**
	Semnan	**1.86 ± 0.18**	**1.80 ± 0.12**	**0.41**
Superoxide dismutase SOD (U/ml)	Damgan	**0.58 ± 0.23**	**0.55 ± 0.14**	**0.31**
	Semnan	**0.55 ± 0.25**	**0.63 ± 0.17**	**0.17**
Glutathione Peroxidase GPx (U/ml/min)	Damgan	**183.5 ± 35.5**	**180.6 ± 64.0**	**0.84**
	Semnan	**181.4 ± 31.8**	**183.7 ± 57.2**	**0.72**
Protein carbonyl (nmol/mg)	Damgan	**2.26 ± 0.59**	**2.14 ± 0.69**	**0.23**
	Semnan	**2.25 ± 0.54**	**2.31 ± 1.64**	**0.46**
Oxidized LDL (µmol/L)	Damgan	**188.9 ± 81.0**	**180.7 ± 64.0**	**0.59**
	Semnan	**198.4 ± 61.5**	**215.4 ± 99.0**	**0.28**
Vitamin A (µgr/dl)	Damgan	**73.2 ± 24.4**	**61.02 ± 19.7**	**0.03**
	Semnan	**73.1 ±24.0**	**62.7 ± 18.2**	**0.02**
Vitamin E (mg/dl)	Damgan	**1.49 ± 0.52**	**1.51 ± 0.53**	**0.83**
	Semnan	**1.52 ± 0.56**	**1.55 ± 0.42**	**0.70**
Lycopene (µgr/dl)	Damgan	**53.1 ± 19.5**	**58.3 ± 25.0**	**0.23**
	Semnan	**64.3 ± 26.4**	**73.2 ± 26.5**	**0.10**
β-carotene (µgr/dl)	Damgan	**26.8 ± 13.3**	**34.7 ± 13.5**	**0.02**
	Semnan	**26.5 ± 12.7**	**37.8 ± 17.1**	**0.01**

Mean values of protein carbonyl and oxidized LDL are shown in table 5-2-7 and table 5-2-8. Both of these oxidative stress biomarkers increased in participants from Semnan (both male and female) after 32 weeks of fortified

bread consumption, but paired t-test showed no significant difference in men and women after 32 weeks compare with baseline values.

Table 5-2-8 Mean and standard deviation of oxidative stress and dietary antioxidants in male participants from Semnan and Damgan at baseline and after 32 weeks

Variables	City	Males (n=60 in Semnan and n=62 in Damgan)		
		Baseline Mean ± SD	After 32 weeks Mean ± SD	P value
Malondialdehyde MDA (µmol/L)	Damgan	**2.9 ± 1.1**	**2.9 ± 0.8**	**0.63**
	Semnan	**3.0 ± 0.9**	**3.2 ± 0.8**	**0.16**
Total antioxidant capacity TAC (µmol/L)	Damgan	**1.87 ± 0.24**	**1.83 ± 0.20**	**0.12**
	Semnan	**1.82 ± 0.21**	**1.76 ± 0.16**	**0.08**
Superoxide dismutase SOD (U/ml)	Damgan	**0.58 ± 0.2**	**0.54 ± 0.1**	**0.24**
	Semnan	**0.59 ± 0.2**	**0.63 ± 0.1**	**0.12**
Glutathione Peroxidase GPx (U/ml/min)	Damgan	**174.6 ± 35.3**	**180.2 ± 63.1**	**0.58**
	Semnan	**178.2 ± 39.2**	**196.8 ± 62.2**	**0.06**
Protein carbonyl (nmol/mg)	Damgan	**2.29 ± 0.27**	**2.13 ± 0.15**	**0.23**
	Semnan	**2.22 ± 0.26**	**2.28 ± 0.18**	**0.38**
Oxidized LDL (µmol/L)	Damgan	**181.8 ± 61.6**	**179.9 ± 85.2**	**0.41**
	Semnan	**185.1 ± 64.2**	**190.5 ± 60.8**	**0.60**
Vitamin A (µgr/dl)	Damgan	**73.3 ± 26.1**	**64.0 ± 13.1**	**0.01**
	Semnan	**78.6 ± 22.6**	**65.6 ± 16.4**	**0.00**
Vitamin E (mg/dl)	Damgan	**1.33 ± 0.51**	**1.46 ± 0.54**	**0.17**
	Semnan	**1.39 ± 0.43**	**1.45 ± 0.37**	**0.20**
Lycopene (µgr/dl)	Damgan	**50.0 ± 21.0**	**53.8 ± 20.9**	**0.39**
	Semnan	**57.5 ± 21.8**	**64.2 ± 22.8**	**0.06**
β-carotene (µgr/dl)	Damgan	**25.0 ± 12.1**	**39.3 ± 16.2**	**0.00**
	Semnan	**27.9 ± 14.1**	**37.6 ± 18.3**	**0.00**

Mean and standard deviation of the serum levels of vitamin A, vitamin E, lycopene, and beta carotene for male and female participants are illustrated in table 5-2-7 and table 5-2-8. These tables show that mean and SD of vitamin A and β-carotene significantly decreased ($p<0.05$) and increased ($p<0.05$) respectively after 32 weeks in male and female participants from the two cities. Paired t-test showed no significant difference in vitamin E and lycopene serum levels of male and female participants from Semnan and Damgan after 32 weeks compared to the baseline.

The total intake of energy, macronutrients, iron, zinc, copper, and antioxidant vitamins of male and female participants at baseline and after 32 weeks are

presented in table 5-2-9 and table 5-2-10. Except for the iron intake that significantly increased among males and females in Semnan after 32 weeks, paired t-test did not show any significant difference during the study (32 weeks) in the intake of these parameters.

Table 5-2-9 Energy, macronutrients, iron, zinc, copper, and antioxidant vitamins intake of the male participants from Semnan and Damgan at baseline and after 32 weeks

Variables	City	Males (n=78 in Semnan, n=87 in Damgan)		
		Baseline Mean(± SD)	After 32 weeks Mean(± SD)	P value
Energy (kcal)	Damgan	**2166.3 ± 488.6**	**2257.5 ± 502.6**	**0.06**
	Semnan	**2207.7 ± 652.8**	**2158.5 ± 600.3**	**0.67**
Protein (gr)	Damgan	**65.4 ± 15.4**	**65.0 ± 16.0**	**0.56**
	Semnan	**66.9 ± 21.5**	**65.7 ± 24.0**	**0.63**
Total fat (gr)	Damgan	**59.5 ± 24.7**	**61.3 ± 25.0**	**0.74**
	Semnan	**66.7 ± 21.0**	**63.3 ± 24.1**	**0.70**
Poly unsaturated fatty acid (PUFA)(gr)	Damgan	**19.4 ± 4.2**	**20.3 ± 4.7**	**0.44**
	Semnan	**22.3 ± 4.9**	**22.9 ± 5.2**	**0.83**
Vitamin C (mg)	Damgan	**88.6 ± 23.0**	**95.4 ± 25.5**	**0.69**
	Semnan	**90.5 ± 26.1**	**93.2 ± 28.3**	**0.62**
Vitamin A(µg)	Damgan	**659.9 ± 297.7**	**661.1 ± 235.0**	**0.85**
	Semnan	**690.5 ± 235.0**	**680.5 ± 220.4**	**0.38**
Vitamin E (mg)	Damgan	**9.1 ± 3.4**	**9.6 ± 3.1**	**0.28**
	Semnan	**9.2 ± 3.5**	**10.4 ± 2.9**	**0.07**
Iron intake (mg)	Damgan	**13.7 ± 4.3**	**13.3 ± 3.8**	**0.63**
	Semnan	**12.9 ± 3.1**	**15.8 ± 6.0**	**000**
Zn intake (mg)	Damgan	**8.34 ± 2.1**	**8.76 ± 2.4**	**0.43**
	Semnan	**7.75 ± 1.8**	**7.71 ± 1.9**	**0.38**
Cu intake (mg)	Damgan	**2.11 ± 0.6**	**1.98 ± 0.7**	**0.24**
	Semnan	**1.87 ± 0.4**	**1.82 ± 0.4**	**0.29**

Except for iron intake, paired t-test did not show any significant differences between the two groups of the study.

Mean and SD of vitamin C and vitamin E (powerful antioxidants), are presented in tables 5-2-9 and 5-2-10. Mean intake of both vitamins increased after 32 weeks in all participants of Semnan and Damgan but this increase was not statistically significant. The RDA of vitamin C for non-smoker adult males and females is 75 and 90 mg/day, respectively. Recommended dietary allowance for vitamin E (alpha-tocopherol) for adults is 15 mg/day.

Table 5-2-9 indicates that the mean intake of vitamin C in females from Semnan and Damgan was more than 90 mg/day which is 20% more than RDA. However, the mean intake of vitamin E in male and female participants from Semnan and Damgan was less than 70% of RDA. It can be concluded that males and females from Semnan and Damgan do not take adequate amounts of vitamin E per day.

Table 5-2-10 Energy, macronutrients, iron, zinc, copper, and antioxidant vitamins intake of female participants from Semnan and Damgan at baseline and after 32 weeks

Variables	City	Females (n=83 in Semnan, n=82 in Damgan)		
		Baseline Mean(± SD)	After 32 weeks Mean(± SD)	P value
Energy (kcal)	Damgan	**1550.8 ± 532.6**	**1685.5 ± 469.5**	**0.34**
	Semnan	**1685.1 ± 455.0**	**1672.5 ± 404.5**	**0.96**
Protein (gr)	Damgan	**50.8 ± 18.7**	**50.1 ± 14.7**	**0.86**
	Semnan	**52.9 ± 16.6**	**50.2 ± 13.9**	**0.48**
Total fat (gr)	Damgan	**52.4 ± 27.5**	**52.3 ± 18.8**	**0.88**
	Semnan	**52.9 ± 22.9**	**50.2 ± 15.5**	**0.29**
Poly unsaturated fatty acid (PUFA)(gr)	Damgan	**18.2 ± 4.0**	**17.3 ± 3.7**	**0.73**
	Semnan	**19.1 ± 4.5**	**16.1 ± 3.6**	**0.12**
Vitamin C (mg)	Damgan	**91.9 ± 33.1**	**97.0 ± 33.0**	**0.65**
	Semnan	**90.6 ± 25.5**	**94.4 ± 24.2**	**0.82**
Vitamin A(µg)	Damgan	**714.7 ± 299.5**	**707.4 ± 283.9**	**0.91**
	Semnan	**686.6 ± 271.0**	**692.2 ± 258.5**	**0.82**
Vitamin E (mg)	Damgan	**8.3 ± 3.1**	**8.9 ± 3.1**	**0.28**
	Semnan	**8.1 ± 3.6**	**8.8 ± 3.0**	**0.15**
Iron intake (mg)	Damgan	**12.1 ± 3.9**	**12.6 ± 3.3**	**0.34**
	Semnan	**11.1 ± 2.8**	**15.3 ± 4.8**	**000**
Zn intake (mg)	Damgan	**7.33 ± 2.2**	**7.38 ± 2.0**	**0.78**
	Semnan	**6.70 ± 1.8**	**6.89 ± 1.9**	**0.82**
Cu intake (mg)	Damgan	**1.89 ± 0.6**	**1.94 ± 0.6**	**0.18**
	Semnan	**1.74 ± 0.5**	**1.70 ± 0.4**	**0.27**

Except for iron intake, paired t-test did not show any significant differences between two groups of the study

Zinc and copper were also important in our study because except for their specific role in oxidative stress, they compete with iron for absorption or intestinal uptake. Recommended dietary allowance for zinc for adult males and females is 11 and 8 mg, respectively. RDA for copper (Cu) is 900 µgr/day. Based on the data presented in tables 5-2-9 and 5-2-10, it can be stated that zinc intake of female participants is much better than male participants, but Cu intake of both males and females in our study were two times more than RDA.

As mentioned earlier, paired t-test did not show any statistically significant differences in these micronutrients between males and females in our study after 32 weeks compared to the baseline.

The total intake of iron in male and female participants from Semnan and Damgan are presented in tables 5-2-9 and 5-2-10. Mean iron intake in male and female participants from Semnan increased significantly which was the result of fortifying flour with 30 mg/kg iron in Semnan for 32 weeks. After starting flour fortification, we measured the iron content of flour regularly (as explained in QA/QC) in flour samples which were taken from bakeries in Semnan and Damgan. Mean iron levels of flour at baseline in Semnan and Damgan were 29.8 and 31.8mg/kg, respectively. The mean iron values in Semnan and Damgan were 61.6 and 33.5 mg/kg throughout the 32 weeks of flour fortification. Based on the iron content of flour in Semnan, which was satisfactory (40-65.9 mg/kg as good level), we can conclude that flour fortification program was successfully implemented in Semnan during the study.

According to the results of this part of the study, paired t-test showed no statistically significant difference in iron status and oxidative stress biomarkers between the study groups after 32 weeks of implementing the flour fortification program in Semnan compared to Damgan.

5-3 Before and after study

Effects of iron fortified flour consumption on iron status and oxidative stress after 64 weeks in Semnan

As mentioned earlier, the governor did not permit us to keep Damgan (control group) without fortification for more than 32 weeks, so we had to start flour fortification in this city. For this reason, in the second part of the study, we did not have Damgan as the control group and therefore, we continued flour fortification with iron in Semnan for 32 weeks again. This part of the study was a before and after study which was conducted in Semnan for 64 weeks. Data collection was done after 64 weeks of starting flour fortification in Semnan for the third time.

Mean and SD of BMI, systolic, and diastolic blood pressure in the three phases of the study, i.e. baseline, after 32 weeks and after 64 weeks, were compared within each group (table 5-3-1). Mean values of BMI, systolic, and diastolic blood pressure of the participants were constant throughout the study for all participants in the two groups. The ANOVA did not show any statistically significant differences in BMI, systolic, and diastolic blood pressure in the three phases of the study within each group (table 5-3-1).

Mean BMI of females was around 30.3 kg/m^2 which indicated that females in our study were overweight or even obese. Compared to females, mean BMI of males was around 26.5 kg/m^2 which showed that most of the men had a normal weight. Based on our data, females in our study were more at risk of oxidative stress than males.

Although mean BMI of the female participants was above normal (BMI between 20 and 25 is defined as normal), their systolic and diastolic blood pressure were around 116 and 78 mmHg respectively that is close to the normal range (The latest medical thinking assumes that a blood pressure of 120 over 80 is the midpoint of the "normal" range).

Table 5-3-1 Means and standard deviation of BMI, systolic, and diastolic blood pressure of male and female participants from Semnan at the beginning, after 32 weeks, and 64 weeks

Variables	Sex	Semnan 53 Males and 51 Females			
		Baseline Mean ± SD	**After 32 weeks** Mean ± SD	**After 64 weeks** Mean ± SD	**P value**
BMI (kg/m^2)	Female	30.3 ± 4.4	30.3 ± 4.3	30.3 ± 4.4	0.99
	Male	26.5 ± 5.5	26.5 ± 5.8	26.7 ± 5.6	0.98
Systolic blood pressure (mmHg)	Female	116.9 ± 14.1	116.3 ± 17.2	116.4 ± 11.5	0.97
	Male	119.8 ± 13.5	115.3 ± 11.9	119.2 ± 12.9	0.16
Diastolic blood pressure (mmHg)	Female	78.6 ± 9.6	77.6 ±10.9	78.3 ± 8.2	0.47
	Male	75.4 ± 8.9	75.3 ± 14.3	76.8 ± 10.7	0.67

The ANOVA did not show significant changes in male and female from Semnan

Table 5-3-2 shows physical activity in both sex groups in Semnan. To compare physical activity in males and females in this part of study, we used the short self- administrated version of IPAQ.

Table 5-3-2 Physical activity of males and females at the beginning, after 32 weeks, and after 64 weeks in Semnan

Sex	Low active %			High active %			P value
	Baseline	After 32 weeks	After 64 weeks	Baseline	After 32 weeks	After 64 weeks	
Female (n=51)	70.6	72.2	70.4	7.8	7.4	7.4	0.7
Male (n=53)	56.0	54.2	54.2	12.0	12.5	10.4	0.9

In Semnan, similar to most parts of Iran, women are less active than men and they spend most of their time indoors. However, males were more physically active than females in our study. Regular physical activity has a low priority to at least half of Iranian men. Those aged between 30 and 50 years are often not very physically active because they are busy with work and family commitments. Lack of physical activity results in a range of health problems including obesity, heart disease, and some forms of cancer. Based on our data, there was no significant difference in regarding physical activity at the beginning, after 32 weeks, and after 64 weeks of the study between males and females.

Mean and SD of iron status including hemoglobin, ferritin, serum iron, TIBC, TS, and serum transferrin receptors in all participants at baseline, after 32, and 64 weeks are compared in Table 5-3-3. In this table, except for serum iron of the male participants, ANOVA did not show any significant differences in other parameters related to iron status at baseline, after 32 weeks (second phase), and after 64 weeks (third phase).

Although mean values of hemoglobin and ferritin increased after 32 and 64 weeks of fortified flour consumption in male and female participants, these changes were not significant (table 5-3-3). The trend of these variables was positive which indicated that with a longer duration of flour fortification, significant changes of these variables would be possible.

Serum transferrin receptor (sTfR) concentration has been recently recognized as a reliable indicator of functional iron deficiency. During this study the amount of serum transferrin receptor was constant, indicating that fortified flour consumption for even 64 weeks or about 8 months had no effects on this precious parameter among non-anemic adults.

Male participants experienced a significant increase in the mean value of serum iron, which describes the concentration of iron (bound to transferrin) in serum, in the third phase (after 64 weeks of fortified flour consumption).

Female participants also experienced an increase in serum iron values after 32 and 64 weeks of the study, but the change was not statistically significant. Significant changes of serum iron in males may be because males ate fortified bread more than females.

Mean TIBC values of males and females in the second and third phase were nearly constant, and there were no obvious changes during the course of the intervention in males and females.

Mean percentage of transferrin saturation (TS) was another parameter of iron status in this study. Comparing values at baseline, in the second phase (after 32 weeks), and the third phase (after 64 weeks) showed that there was an increase in the percentage of TS in both males and females during the study. Although ANOVA showed no significant difference between the study groups, the positive trend of TS in the second and third phase showed that transferrin

saturation was increasing. So, if fortification continued for a longer period, significant changes would possible.

Table 5-3-3 Mean and standard deviation of iron status and serum endogenous antioxidant in male and female participants from Semnan at baseline, after 32 weeks and after 64 weeks

Variables	Sex	Semnan 53 Male and 51 Female			
		Baseline Mean ± SD	After 32 weeks Mean ± SD	After 64 weeks Mean ± SD	P value
Hemoglobin (gr/dl)	Female	12.8 ± 0.6	12.8 ± 0.7	13.2 ± 0.9	0.33
	Male	14.2 ± 0.7	14.3 ± 0.8	14.5 ± 0.8	0.14
Ferritin (ngr/ml)	Female	98.0 ± 43.9	104.0 ± 52.0	104.8 ± 35.0	0.72
	Male	143.0 ± 65.0	146.1 ± 72.8	168.0 ± 40.3	0.10
Serum transferrin receptor (µgr/ml)	Female	1.3 ± 0.34	1.3 ± 0.40	1.3 ± 0.46	0.87
	Male	1.2 ± 0.5	1.1 ± 0.5	1.2 ± 0.4	0.79
Serum Iron (µgr/dl)	Female	90.5 ± 29.4	94.5 ± 29.5	97.1 ± 26.2	0.50
	Male	102.9 ± 31.5	109.7 ± 31.6	117.2 ± 29.8[ab]	0.00
Total Iron Binding Capacity (TIBC) (µgr/dl)	Female	301.4± 75.6	299.1 ± 83.0	295.8 ± 49.6	0.52
	Male	327.1 ± 78.8	320.8 ± 64.8	332.9 ± 37.3	0.43
Transferrin Saturation (TS) (%)	Female	28.3 ± 9.9	28.6 ± 8.4	29.2 ± 8.5	0.87
	Male	30.5 ± 9.3	31.5 ± 10.5	33.8 ± 8.8	0.32
Albumin (gr/dl)	Female	4.0 ± 0.3	4.1 ± 0.6	5.0 ± 0.3[ab]	000
	Male	3.9 ± 0.3	4.2 ± 0.4	5.1 ± 0.4[ab]	000
Total Bilirubin (µgr/d)	Female	0.85 ± 0.32	0.92 ± 0.34	0.82 ± 0.16	0.10
	Male	1.11 ± 0.48	0.87 ± 0.23[a]	0.93 ± 0.23	0.05
Total protein (gr/dl)	Female	7.0 ± 0.4	7.3 ± 0.6	7.8 ± 0.7[ab]	000
	Male	6.9 ± 0.5	7.4 ± 0.6	7.7 ± 0.7[a]	000
Uric acid (mg/dl)	Female	4.6 ± 1.1	4.4 ± 1.1	4.3 ± 1.1	0.53
	Male	5.0 ± 1.2	5.1 ± 1.2	5.0 ± 1.1	0.85

a: compared with the first phase, b: compared with the second phase

Mean and SD of albumin, total bilirubin, uric acid, and total protein in the three phases of the study were compared within each group (table 5-3-3). ANOVA did not show significant changes in uric acid throughout the study for males and females but showed a significant difference in serum albumin of the subjects within each group at the three intervals of the study. In order to compare the third phase with the second and the first phase, multiple comparisons using Bonferroni and Dunnett post-hoc tests were utilized. Multiple comparisons showed that mean values of albumin at the third phase in both male and female participants significantly increased when compared to the second and the first phase ($p<0.001$).

Total protein significantly increased ($p<0.001$) in females in the third phase (after 64 weeks), compared to the second (32 weeks) and the first phase (baseline). In males, this increase was only significant when comparing the third phase (after 64 weeks) with the first phase (baseline).

Albumin comprises about half of the blood serum protein. Significant changes of total protein and albumin at the third phase confirm each other.

ANOVA did not show any statistically significant differences in total bilirubin in females. However, ANOVA showed significant changes in total bilirubin in males when comparing the first phase with the second phase (baseline and after 32 weeks).

Mean and SD of oxidative stress biomarkers (including; MDA, TAC, SOD, GPx, PC, Ox-LDL) at baseline, after 32 weeks and after 64 weeks are presented in table 5-3-4. ANOVA showed that in males, mean TAC decreased and mean SOD and GPX increased significantly after 64 weeks ($p<0.05$). ANOVA did not show significant changes in these parameters after 64 weeks in females.

Comparing the mean values of MDA and Oxidized-LDL at baseline with the second phase (after 32 weeks) and the third phase (after 64 weeks) showed that there was an increase in the amount of MDA and Oxidized-LDL in both males and females. Although this increase was not significant, it indicated that if flour fortification continued for a longer time, significant changes in these parameters would probable.

Mean and SD of TAC in males decreased in the second (after 32 weeks) and the third phase (after 64 weeks) of the study. Multiple comparisons showed that the decrease of TAC was only significant when the third phase was compared with the first phase.

Comparing TAC values of females in third phase with the second and the first phase was also interesting. TAC values of females in the first and the second phase was constant but decreased after 64 weeks of fortified flour consumption. ANOVA did not show any significant differences in mean TAC of the female participants at the three intervals of the study.

Mean values of SOD and GPx for both males and females are illustrated in table 5-3-4. The values of these two oxidative stress parameters, which are important enzymes in protecting body from oxidants, increased in the second (after 32 weeks) and the third phase (after 64 weeks) of the study in both genders. ANOVA showed that the changes in SOD and GPx were statistically significant only in males ($p<0.02$ for SOD, $p<0.04$ for GPx) which was related to the amount of these parameters in the third phase (after 64 weeks) as shown in the table 5-3-4. In fact, multiple comparisons showed that there were no significant differences in SOD and GPx mean values in the second and the first phase in males and females.

Mean and SD of protein carbonyl (PC) at the three intervals of the study are presented in table 5-3-4 as well. ANOVA showed no significant differences in PC between groups at baseline, after 32 weeks, and after 64 weeks of fortified flour consumption. The mean values of PC at the third phase decreased insignificantly when compared to the second phase of the study in males and females.

Mean and SD of serum vitamin E, Lycopene, β-carotene, and vitamin A are also presented in table 5-3-4. ANOVA showed no statistically significant differences in lycopene between groups in the three phases of the study but showed that mean serum levels of vitamin A in both genders decreased significantly in the three phases of the study ($p<0.05$) (table 5-3-4).

Table 5-3-4 Mean and standard deviation of oxidative stress and dietary antioxidants in male and female participants from Semnan at baseline, after 32 weeks, and after 64 weeks

Variables	Sex	Semnan 53 Males and 51 Females			
		Baseline Mean ± SD	After 32 weeks Mean ± SD	After 64weeks Mean ± SD	P value
Malondialdehyde, MDA (µmol/L)	Female	2.9 ± 1.1	3.0 ± 1.0	3.2 ± 0.7	0.48
	Male	3.0 ± 1.0	3.1 ± 0.8	3.2 ± 0.7	0.36
Total antioxidant capacity, TAC (µmol/L)	Female	1.77 ± 0.16	1.77 ± 0.12	1.73 ± 0.10	0.18
	Male	1.83 ± 0.17	1.78 ± 0.14	1.71 ± 0.10[a]	0.01
Superoxide dismutase , SOD (U/ml)	Female	0.55 ± 0.25	0.63 ± 0.10	0.65 ± 0.12	0.07
	Male	0.56 ± 0.24	0.62 ± 0.10	0.67 ± 0.12[a]	0.02
Glutathione Peroxidase, GPx (U/ml/min)	Female	181.4 ± 48.1	193.7 ± 50.8	194.1 ± 59.1	0.11
	Male	176.6 ± 38.1	192.0 ± 49.3	200.0 ± 46.7[a]	0.04
Protein carbonyl , PC (nmol/mg)	Female	2.23 ± 0.51	2.37 ± 1.34	2.34 ± 1.27	0.48
	Male	2.21 ± 0.58	2.27 ± 1.4	2.18 ± 1.2	0.33
Oxidized LDL (µmol/L)	Female	199.1 ± 64.1	215.0 ± 87.8	226.9 ± 89.0	0.31
	Male	185.1 ± 64.2	193.6 ± 65.0	201.0 ± 70.6	0.50
Vitamin A (µgr/dl)	Female	72.7 ± 24.0	63.0 ± 23.1[a]	58.7 ± 19.3[a]	000
	Male	78.6 ± 22.6	66.0 ± 16.4[a]	62.8 ± 14.1[a]	000
Vitamin E (mg/dl)	Female	1.53 ± 0.6	1.55 ± 0.4	1.57 ± 0.4	0.90
	Male	1.39 ± 0.4	1.46 ± 0.3	1.64 ± 0.4[a]	0.01
Lycopene (µgr/dl)	Female	64.1 ± 35.9	72.0 ± 26.5	64.5 ± 24.8	0.31
	Male	57.5 ± 21.9	64.0 ± 22.6	66.4 ± 19.4	0.11
β-carotene (µgr/dl)	Female	27.3 ± 18.9	38.5 ± 23.7[a]	35.6 ± 18.8	0.03
	Male	28.8 ± 13.3	36.5 ± 16.5	38.8 ± 16.2[a]	0.02

a: compared with the first phase, b: compared with the second phase

The mean values of serum vitamin E in study groups as shown in table 5-3-4 increased in the three phases of the study but this increase was only significant among males ($p<0.05$).

Male participants experienced a significant increase in mean values of serum β-carotene in the three phases of the study ($p<0.05$). For females, however, the difference was significant when the second phase was compared with the first phase.

The total intake of energy, macronutrients, iron, zinc, copper, and antioxidant vitamins in male and female participants at baseline, after 32 weeks, and after 64 weeks of implementing flour fortification are presented in table 5-3-5. Except for iron intake, ANOVA showed no statistically significant difference between groups in the three phases of the study.

Mean total energy intake of males and females in this part of the study was nearly constant; however, it decreased insignificantly in the third phase.

Comparison between baseline and the third phase (after 64 weeks fortified flour consumption) showed that there was an increase in the amount of total protein intake in both genders (table 5-3-5). There was a decrease in the amount of total fat intake as well. The amount of PUFA decreased in females while it increased in males. The mean intake of vitamin C increased in both genders at the end of the study compared to the baseline. Mean values of vitamin A in females increased insignificantly.

Table 5-3-5 Energy, macronutrients, iron, zinc, copper, and antioxidant vitamins intake of males and females at the baseline, after 32, and 64 weeks in Semnan

Variables	Sex	Semnan 53 Males and 51 Females			
		Baseline Mean ± SD	After 32 weeks Mean ± SD	After 64 weeks Mean ± SD	P value
Energy (kcal)	Female	1693.1 ± 460.0	1677.5 ± 424.5	1660.8 ± 456.6	0.93
	Male	2207.7 ± 652.8	2164.5 ± 600.3	2177.3 ± 705.1	0.67
Protein (gr)	Female	52.7 ± 16.3	51.2 ± 13.9	53.0 ± 15.9	0.58
	Male	65.7 ± 20.6	63.7 ± 24.0	72.1 ± 23.8	0.35
Total fat (gr)	Female	53.9 ± 21.5	51.2 ± 15.5	53.4 ± 24.5	0.76
	Male	69.7 ± 23.2	64.3 ± 22.1	68.5 ± 22.3	0.66
Poly unsaturated fatty acid (PUFA)(gr)	Female	19.3 ± 4.5	16.4 ± 3.6	18.2 ± 4.1	0.32
	Male	22.8 ± 4.9	23.9 ± 5.2	25.8 ± 5.4	0.42
Vitamin C (mg)	Female	92.6 ± 27.5	95.4 ± 25.2	98.7 ± 27.7	0.53
	Male	89.5 ± 27.1	92.2 ± 28.3	97.5 ± 30.1	0.62
Vitamin A(µg)	Female	680.6 ± 273	689.2 ± 248.5	682.8 ± 223.4	0.82
	Male	694.5 ± 238	687.5 ± 223	671.5 ± 225	0.28
Vitamin E (mg)	Female	8.8 ± 3.7	9.5 ± 3.3	9.9 ± 2.3	0.15
	Male	9.3 ± 3.2	10.6 ± 2.9	10.9 ± 3.9	0.25
Iron intake (mg)	Female	11.3 ± 2.9	15.5 ± 4.3	14.7 ± 4.0	000
	Male	12.5 ± 3.1	15.4 ± 5.9	15.6 ± 5.0	000
Zn intake (mg)	Female	6.81 ± 1.8	6.94 ± 1.8	6.72 ± 1.7	0.33
	Male	7.83 ± 1.9	7.78 ± 2.0	7.71 ± 1.9	0.56
Cu intake (mg)	Female	1.77 ± 0.6	1.72 ± 0.5	1.69 ± 0.5	0.44
	Male	1.91 ± 0.4	1.86 ± 0.5	1.81 ± 0.4	0.65

Mean intake of vitamin E increased in both genders at the third phase (after 64 weeks). The mean intake of zinc (Zn) and copper (Cu) decreased in males and females at the end of study or after 64 weeks of consuming fortified flour. None

of the above-mentioned changes were statistically significant in the three phases of the study (baseline, after 32 weeks and after 64 weeks).

As a result of consuming fortified bread in Semnan during the study, the total mean intake of iron increased in study groups. ANOVA showed statistically significant differences ($p<0.01$) in males and females in the three phases of study (table 5-3-5).

As mentioned earlier, one of the main parts of our study was QA/QC to monitor flour fortification with iron. For this reason, regular flour samples from three factories and bakeries of Semnan were collected and sent them to an accredited laboratory to measure the iron content of flour.

For the process evaluation, we first measured the iron content of flour samples from two factories in Semnan after fortification (Omide Semnan and Ardvasabose Semnan).
Mean iron level of flour during the first 32 weeks of flour fortification was 54.6 mg/kg in 39 samples from Omide Semnan and 57.8 mg/kg in 33 samples from Ardvasabose Semnan. During the second 32 weeks of flour fortification, the mean level of iron was 57.9 mg/kg in 29 flour samples from Omide Semnan and 60.1 mg/kg in 31 flour samples from Ardvasabose Semnan. These iron levels are defined as 'good' (40-65.9 mg/kg).
Table 5-3-6 shows iron distribution in flour samples obtained from bakeries during the study. This table shows the exact amount of iron in flour based on spectrophotometric measurements. According to this table, flour fortification was successfully implemented in Semnan.

Table 5-3-6 Iron content (mg/kg) of flour samples obtained from bakeries at baseline, within the first 32 weeks, and within the second 32 weeks of the study

City	Baseline Median(IQR)	Within first 32 weeks Median(IQR)	Within second 32 weeks Median(IQR)
Semnan	29.8 (9.10) n=32	61.6 (13.8) n=29	59.6 (12.9) n=28

6 DISCUSSION

6-1 Baseline characteristics of the subjects

In the present study, at baseline, there was no significant difference in age, BMI, systolic and diastolic blood pressure between males and females in two cities. Mean BMI of males was nearly in the normal range in both cities but women had a mean BMI of more than 30 kg/m^2 which is categorized as obese. However, obesity was a very important concern in our study. Independent t-test showed no significant difference in women in the two cities. Obesity can independently influence the results and can act as a confounding factor.

Gender is a significant effect modifier in the association between adiposity and oxidative stress. The reasons for different results between genders regarding adiposity and oxidative stress are not clear and require additional investigation.

Physical activity is also a significant effect modifier in the association between adiposity and oxidative stress. Results of this part showed that women are less active than men but there was no statistically significant difference within each gender at the start of the study.

One of the recent studies on the association between obesity and oxidative stress is a cross sectional study by Charles et al (Charles et al, 2008). In this study, adiposity measures and oxidative stress were evaluated among police officers. This study included randomly selected police officers (43policewomen; 67 policemen) from Buffalo, New York. Officers were 26- to 61-years old. Adiposity measures were evaluated using standardized methods. Biomarkers were measured on fasting blood specimens. An oxidative stress score (OSS) was created as a composite of the biomarkers. Results of this study showed that GPx was inversely associated with BMI (trend $p = 0.004$) and with waist-to-height ratio (trend $p = 0.017$). The results of this study showed that adiposity was significantly associated with several markers of oxidative stress and lower antioxidant defense.

In another study by Amirkhizi et al (Amirkhizi et al, 2007), the hypothesis that obesity increases plasma lipid peroxidation and decreases erythrocyte cytoprotection was tested. In this study, 160 obese women (BMI=30-40 Kg/m^2) aged 20-45 years were randomly selected. The concentration of plasma MDA was significantly higher ($P<0.001$) in obese women compared to women with healthy BMI. Furthermore, there was a significantly positive correlation ($r =0.75$, $P<0.0001$) between BMI and plasma MDA. On the other hand, women with a healthy BMI had a significantly higher ($P<0.001$) erythrocyte SOD and GPX activity than obese women. Furthermore, erythrocyte SOD and GPX activity was negatively correlated with BMI ($r =-0.52$, $P<0.0001$ and $r =-0.42$, $P<0.001$), respectively. No significant difference was observed between the two groups in erythrocyte CAT activity. It is concluded that obesity, even in the absence of smoking, diabetes, renal or liver diseases, can decrease the activity of body's protective antioxidants, and can enhance the systemic oxidative stress.

We did not find any significant changes in energy, protein, fat, and other relevant nutrient intakes in participants at the baseline of the study. At the three intervals (baseline, after 32 weeks, after 64 weeks) a 3-day 24-hour dietary recall and quantitative food frequency questionnaire were completed for each participant by a trained nutritionist. Quantities were estimated from photographs of portion sizes and from household measures (Ghafarpour et al, 2007). Participants were instructed to maintain their usual dietary habits throughout the study. Monthly follow-ups were performed by phone.

Bread is a staple food in Iran and the mean intake of bread in Semnan is about 300gr/day. Flour fortification, when appropriately implemented, does not significantly affect the organoleptic qualities (color, smell or taste) of foods such as cakes, breads, and noodles. Therefore, flour fortification and fortified bread consumption in the diet of the subjects did not change their dietary habits.
There were no significant differences in iron status and oxidative stress biomarkers at the baseline of the study. It is possible that by introducing fortified bread to the diet of subjects, changes in iron status or consequently oxidative stress ensue.

6-2 The first hypothesis

Flour fortification program does not cause iron overload in healthy non-anemic adults after 32 and 64 weeks

Iron overload is defined as the accumulation of iron in the body due to any cause. Iron overload is a serious chronic condition that must be properly diagnosed and treated. Undiagnosed iron overload can lead to hemochromatosis, which is potentially life-threatening.

Transferrin saturation and serum ferritin were used to determine iron overload in our study.

In this regard, ferritin levels more than 200 ng/ml for female subjects and more than 300 ng/ml for male subjects and transferrin saturation higher than 55% for both genders were considered as iron overload.

In table 6-2-1, the number of the subjects that were diagnosed with iron overload based on both ferritin and transferrin saturation in Semnan are presented. The following table (table 6-2-1) even shows that the number of iron overload subjects did not changed during the study and the change in the rates is just due to the decrease of the number of the participants in the second (after 32 weeks) and the third (after 64 weeks) phase of the study.

Regarding the results in table 6-2-1, there were no significant changes in ferritin and transferrin saturation in men and women. Therefore, flour fortification did not cause iron overload after 32 and 64 weeks.

Fortifying flour with iron has been used as an efficient and inexpensive approach for reducing the prevalence of iron deficiency in many countries. However, this method has been abolished, even in some developed countries, due to the probable negative effects of iron overload (Olssen et al, 1997).

Table 6-2-1 The number of iron overload subjects out of total number of each groups based on ferritin more than 200 ng/ml for women and more than 300 ng/ml for men and transferrin saturation more than 55 % for both sex in Semnan

sex	Baseline Num (out of)	After 32 weeks Num (out of)	After 64 weeks Num (out of)
Female	3 (78) 3.8%	3 (60) 5%	3 (53) 5.6%
Male	4 (83) 4.8%	4 (66) 6.1%	4 (51) 7.8%

In the recent 10 years, several studies have been done on this issue but unfortunately, their results are not complete and do not yield a clear conclusion. For example, although the relationship between serum ferritin and the risk of cardio-vascular diseases has been reviewed in at least 14 studies, result of a meta-analysis could not definitely confirm the relationship (Danesh and Appleby, 1999).

Concerns regarding the negative effects of iron have been raised after considerable progresses were made in recognizing iron metabolism and its role in developing active oxygen (Hentze et al, 2004). The highest tolerable iron intake is 45 mg/day (Allen et al, 2006). Although it is nearly impossible to reach this level by adding 30 mg iron to one kg of flour, there is evidence of appearing iron related disorders with even lower doses in the long term (Rehema et al, 1998).

Because of continuous blood transfusions, thalassemia patients are prone to peroxidative tissue injury through secondary iron overload. In a study by Livrea et al (Livrea et al, 1996), oxidative stress in thalassemia patients was investigated. In this study, analysis of serum from 42 β-thalassemia patients, aged 4 to 40 years, showed that the mean concentrations of MDA, PC, and conjugated diene increased about twofold compared to the control. Ferritin levels were positively correlated with the amount of MDA ($r = 0.41$; $p= 0.007$) and showed a positive trend with protein carbonyl ($r = 0.35$; $p= 0.07$) as further evidence for deleterious effects of high tissue iron levels. The total serum

antioxidant capacity also significantly decreased by 14%. Serum levels of vitamin E were inversely correlated with ferritin ($r = -0.45$; $p=0.003$), suggesting a major consumption of this antioxidant under iron overload. These results suggested that iron-induced liver damage in thalassemia may play a major role in depleting lipid-soluble antioxidants.

In a study by Day (Day et al, 2003) on mice, giving 15 mg/day iron to mice parenterally for 6 weeks caused iron overload and increased the production of ROS, and the incidence of cardio-vascular diseases. Although the relationship between iron overload and cardio-vascular diseases in animals cannot be generalized to humans with certainty, the results of this study showed the specific role of iron in developing cardio-vascular diseases and thrombosis.

Although in studies by livrea (Livrea et al, 1996) and Day (Day et al, 2003), a close relationship was reported between iron intake or injected iron and iron overload, it seems that our study is more similar to Hallberg's study which concluded that iron overload was not probable if fortified foods were consumed due to the change of iron absorption proportionate to the amount of iron intake (Hallberg et al, 1997) (Hallberg et al, 2002).

One of the noticeable and similar studies to our research is a study by Rehema et al (Rehema et al, 1998). In this study, effects of long-term alimentary (drinking water) iron overload on the parameters of oxidative stress were evaluated. The study group ($n = 35$) from a village in southern Estonia was 37.1 ± 13.3 years old, and the mean period of drinking water iron overload was 20.6 ± 9.3 years. Daily consumption of alimentary iron was 25±10mg, 10±4mg of that from drinking water. Results of this study showed that the mean value of serum iron was significantly higher than normal. The total iron-binding capacity of serum tended to lower. The parameters of lipid peroxidation, such as conjugated dienes and thiobarbituric acid reactive substances, also significantly increased ($p<0.001$). The total antioxidant capacity of serum did not change. It can be concluded that long-term alimentary iron overload results in a positive serum iron balance which in turn increases oxidative stress.

The similarity of our study with this study is in the absence of anemia and iron deficiency in subjects. Moreover, the amount of iron was similar to our study (9 mg/day in our study compared to 10 mg/day in Rehema's study) but Rehema's study was continued for a longer period (20 years) than ours (64 weeks or about 16 months). However, it is not clear whether Rehema reached the same results if the duration of the study was shorter. As we used the same amount of daily iron intake as Rehema did, it could be concluded that long-term flour fortification results in iron overload in non-anemic healthy individuals.

Evidence suggests that high iron intake and high levels of iron store are risk factors of cardio-vascular diseases and cancer.

In this study, transferrin and ferritin as markers of detecting iron overload did not show iron overload in participants after 32 and 64 weeks of intervention but there was a positive trend in nearly all parameters of iron status which is suggestive of increased iron retention in the body. Therefore, it is possible that consuming fortified bread by non-anemic individuals for a longer period causes iron overload as a result of increased iron retention.

6-3 The Second hypothesis

Consuming Iron fortified flour does not induce oxidative stress biomarkers in the study population after 32 and 64 weeks

This hypothesis is divided into two parts:

6-3-1 Consuming Iron fortified flour does not induce oxidative stress biomarkers in the study population after 32 weeks

6-3-2 Consuming Iron fortified flour does not induce oxidative stress biomarkers in the study population after 64 weeks

6-3-1 Consuming Iron fortified flour does not induce oxidative stress biomarkers in the study population after 32 weeks:

This part of the study was done through a field trial. After baseline data collection, flour fortification started with 30 mg/kg iron in Semnan. Based on mean bread consumption in Semnan (300 gr/day), 9 mg iron was added to their daily food baskets. So, subjects of Semnan (intervention group) consumed bread baked with fortified flour for 32 weeks (about 8 months) while subjects of Damgan (control group) consumed bread with non-fortified flour in this period.

It should be noted that since different kinds of breads are baked in these two cities, for ensuring the consumption of iron fortified bread by all subjects of Semnan, all bakery flour of this city was fortified during the study. Meanwhile, in addition to continuous monitoring of the iron content of the fortified flour, the research team regularly collected flour samples from bakeries of Semnan and Damgan and sent them to an accredited laboratory for determining their iron content. The results showed that the bread baked in Semnan was fortified and the bread baked in Damgan was not fortified and did not contain the added amounts of iron. Also, tables 5-2-9 and 5-2-10 show that fortification program

significantly increased the iron content of consumed bread of the male and female participants of Semnan ($p<0.001$) during the study. The iron intake of the male and female participants of Damgan did not significantly change in this period. These findings show that the flour fortification program was successfully implemented in Semnan (intervention group).

In the field trial part of our study, after 32 weeks of fortified flour consumption, mean values of hemoglobin, ferritin, serum iron, TIBC, TS, and serum transferrin receptor did not show any statistically significant differences between the study groups in Semnan compared to Damgan. Comparing changes in the mean differences of oxidative stress biomarkers such as MDA, TAC, SOD, GPx, protein carbonyl, and oxidized-LDL in Semnan and Damgan also did not show any significant differences.

In some studies, in which iron was given to the subjects, some oxidative stress biomarkers significantly changed.

In a double-blind randomized clinical trial study by Khoshfetrat et al (Khoshfetrat et al, 2008), oxidative stress indices were compared between iron deficient and healthy subjects. In this study, 60 non-anemic iron deficient and 30 healthy female students were selected. After matching, non-anemic iron deficient students were randomly assigned into two groups receiving 50 mg/day elemental iron with and/or without 500 mg/day ascorbic acid for 12 weeks. At the end of the study, serum TAC significantly increased in supplemented subjects, not only compared to the baseline values (within group) but also in comparison with controls (between groups) ($p<0.001$). The mean MDA concentration in the group receiving both iron and vitamin C was significantly less than group receiving iron alone. It seems that iron supplementation with ascorbic acid can decrease the oxidative stress so it can be recommended for more efficient control of iron deficiency.

In a clinical randomized trial by Amirkhizi (Amirkhizi et al, 2006), effects of iron supplementation on lipid peroxidation among iron deficient women aged 20 to 45 year were investigated. In this study, 38 non-anemic iron deficient and 36 healthy women were randomly selected. Iron supplementation consisting of 120 mg ferrous sulfate (50 mg elemental iron) was given to iron deficient non-

anemic women. Results of this study showed that iron supplementation increased lipid peroxidation and also decreased total antioxidant capacity.

Amirkhizi et al (Amirkhizi et al, 2008) investigated any relationship between body iron stores with levels of oxidative stress in another study. The results showed that subjects in the highest tertile of plasma ferritin had the highest levels of plasma MDA ($p<0.001$) and catalase activity ($p<0.05$). These findings revealed an association between body iron stores and oxidative stress markers.

In spite of the studies that showed the positive correlation between iron and oxidative stress, there are a few studies that have failed to show any significant effect of iron on oxidative stress.

In a study by Gropper et al (Gropper et al, 2003), giving 50 mg iron to non-anemic iron deficient girls for two months improved their iron status and increased their iron reserves but did not affect oxidative stress indicators significantly. Also, Binkoski et al (Binkoski et al, 2003) in a study showed that iron supplementation in iron deficient women improved their iron status without increasing their oxidative stress. In this study, giving 100mg/day iron to healthy and iron deficient women for eight weeks enhanced their serum ferritin status but did not change their oxidative stress indicators significantly. The difference between studies by Binkoski and Gropper and our study is that our intervention time was longer but the amount of iron intake in our study was much less than those two studies (50mg/day iron in the study by Gropper et al and 100mg/day iron in the study by Binkoski et al compared to 9mg/day iron in our study).

Iron deficiency does not only affect the production of hemoglobin, but also the production of other proteins containing Fe^{2+} such as cytochromes, myoglobin, catalase, and peroxidase. The extracellularly generated O_2•- and H_2O_2 have been shown to traverse erythrocyte membranes. Impairment of antioxidant defense system and reduced cellular immunity and myeloperoxidase activity were previously reported in patients with iron deficiency anemia. All of these factors may contribute to inadequate erythrocyte survival.

Erythrocytes are excellently equipped to handle intracellular oxidative stress through combined activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione (GSH). However, the protective efficiency of erythrocytes against lipid peroxidation and carbonylation depends on the balance between oxidant species and the availability of antioxidant defenses. Literature offers contradictory and limited data on oxidative stress and antioxidant defenses in patients with iron deficiency anemia.

In a study by Kurtoglu et al (Kortoglu et al, 2003), the effect of iron supplementation on antioxidant status in iron-deficient anemia, including the time for hemoglobin normalization and at the time of filling iron body stores was measured. The extent of plasma lipid peroxidation was evaluated by measuring the levels of malondialdehyde and glutathione peroxidase (GPx), and the activity of superoxide dismutase (SOD) and catalase in 63 patients with iron-deficiency anemia before and after 6 weeks of iron supplementation and when body iron stores were saturated. After 6 weeks of iron supplementation, a significant decrease in oxidative stress was observed in the treated subjects compared to controls ($p<0.05$). No significant difference existed between treated patients after 6 weeks and at the end of the study. The erythrocyte levels of catalase, SOD, and GPx were significantly lower in treated patients compared to controls ($p<0.05$). These levels increased after 6 weeks of supplementation ($p<0.05$) and showed no significant difference with the values at the end of the study. These results indicated that iron supplementation did not induce oxidative stress. It was also concluded that to evaluate the oxidative stress and iron supplementation, time is important.

Appropriate adjustment of iron absorption from the digestive system and inhibiting the entrance of surplus iron can be other reasons mentioned in the study by Hallberg et al (Hallberg et al, 1997). Hallberg et al showed the effect of ferritin on inhibiting iron absorption from the digestive system and even mentioned that in amounts of ferritin higher than 60ng /ml, iron absorption from the digestive system decreased and therefore, in iron fortification programs, increase of the iron absorption is not a concern. In our study, the average of ferritin in both genders was higher than 60ng/ml that is completely compatible with the results of the Hallberg's study. However, it is still debated whether the

ability of human body in controlling iron absorption can persist for a long time. This question is partly answered in the next part of our study.

The reason why no significant changes were observed in iron status and oxidative stress after 32 weeks of fortified flour consumption within each gender could be that our participants were non-anemic and healthy people. The second reason could be the iron doses which were several times more than the amount which was in our study.

Time is also important, positive trend in some oxidative stress biomarkers such as SOD, GPx, protein carbonyl, and oxidized-LDL can be the first step of significant changes if flour fortification continues for a longer duration.

Totally, according to results of our field trial and comparing them with the results of other studies, implementing flour fortification for 32 weeks does not have any adverse effects on oxidative stress indicators and iron status among non-anemic healthy men and women.

6-3-2 Consuming Iron fortified flour does not induce oxidative stress biomarkers in the study population after 64 weeks:

If we had Damgan as the control group for the second 32 weeks too, our study would be more comprehensive. Due to the objection of the governor and other authorities of Semnan province to postponing flour fortification in Damgan, we had to start this program in Damgan.

In order to study the effects of flour fortification for 64 weeks, flour fortification was continued for another 32 weeks in Semnan and data of the third phase (after 64 weeks) of Semnan was compared with the information of the first phase as a before and after study.

After testing the normal distribution of variables by Kolmogrov-Smirnov test, one-way ANOVA was used for comparing averages among different groups. Multiple comparisons among phases were done with the Bonferroni and Dunnett post-hoc tests. It should be noted that the volunteers who had

participated in the first phase of the study, participated in the second and third phases too (the target population was paired before and after intervention).

To study the changes in oxidative stress indicators, undertaking the correct process of flour fortification program in Semnan had to be ensured in the first step. In fact, we had to make sure that flour fortification program was well-implemented and based on the determined objectives in the second 32 weeks in Semnan. In this regard, during the second phase of the study, twenty eight flour samples from bakeries in Semnan were analyzed. The median (IQR) iron concentration of flour was 61.6 (13.8) mg/kg, which was defined as 'good' (40-65.9 mg/kg). Also sixty five flour samples from two flour factories (Omid Semnan and Ardvasaboose Semnan) in Semnan were analyzed by Central Food and Nutrition Lab (CFNL). Mean (±SD) iron level of flour was 54.7 (11.2) mg/kg, which was defined as 'good'.
The amount of iron in flour and bread showed the successful implementation of fortification program in Semnan and it was indicated that the program was successfully implemented until the end of the study.

The mean and SD of iron status and oxidative stress biomarkers were measured after 64 weeks. Although the trend of haemoglobin, ferritin, and transferrin saturation was positive in both genders, except for serum iron in male participants, ANOVA did not show any statistically significant differences in the rest of iron status parameters in the three phases of the study in males and females (table 5-3-3).

Also, among oxidative stress biomarkers, male participants experienced a significant change in mean serum levels of SOD ($p<0.001$) and GPx ($p<0.05$) activity in third phase (after 64 weeks) compared to baseline (table 5-3-4). The positive and significant correlation between serum iron and SOD in male subjects of Semnan was seen in the third phase of the study, too ($p<0.05$, $r = 0.249$).

Heinecke et al (Heineke et al, 1984) in an in vivo study on smooth muscle cells derived from explants of human aorta showed that Fe and Cu content of the incubation medium profoundly influenced modification of LDL by human arterial smooth muscle cells. Modification, as assessed by the increase in thiobarbituric

acid reacting substances (TBARS) and in electrophoretic mobility, was promoted by micromolar concentrations of either Fe or Cu.

Carrier et al (Carrier et al, 2002) designed a study to determine whether oral iron supplementation in rats with dextran sulfate sodium (DSS)-induced colitis increased intestinal inflammation and oxidative stress and whether the addition of an antioxidant, vitamin E, reduced this detrimental effect. Results of this study showed that oral iron supplementation significantly enhanced disease activity in rats with DSS-induced colitis which was associated with an increase in oxidative stress. Vitamin E, a potent antioxidant, did not reduce lipid peroxidation but significantly reduced intestinal inflammation and disease activity, even with concurrent iron supplementation.

In a population-based case-control study by Kallianpur et al (Kallianpur et al, 2008) the effects of iron and fats from various food sources on the risk of breast cancer were investigated. In this study, 3452 breast cancer cases, and 3474 age-frequency matched controls completed an in-person interview including a detailed food-frequency questionnaire. Results of this study showed that a high intake of animal-derived iron (heme iron) might be associated with an increased risk of primary breast cancer in Chinese women, and saturated and mono-unsaturated fats derived from animal sources might augment this effect.

In another study by Bae et al (Bae et al, 2009), the relationship between iron and oxidative stress, as potential risk factors, and the development of breast cancer (BC) was investigated. BC patients (n = 121) and healthy age-matched controls (n = 149) entered the study. Iron and antioxidant vitamins intakes were estimated using a quantitative food frequency questionnaire. This study showed that total and non-heme iron intake of BC patients were lower than those of the controls. However, the serum iron level was significantly higher in BC patients. Plasma MDA levels were also significantly higher in BC patients. These results suggested that serum iron overload could be a breast cancer risk factor, possibly due to increased oxidative stress.

Epidemiological evidence concerning the role of iron, a lipid peroxidation catalyst, in coronary heart disease (CHD) is inconsistent. Tuomainen

(Tuomanain et al, 1998) investigated the association between the concentration ratio of serum transferrin receptor to serum ferritin (TfR/ferritin) and the risk of acute myocardial infarction (AMI). In this prospective nested case-control study on eastern male Finns, results showed an association between increased body iron stores and increased risk of AMI, confirming previous epidemiological findings.

Ferritin is a 24 subunit protein composed of two subunit types, termed H and L. the ferritin H subunit has a potent ferroxidase activity that catalyses the oxidation of ferrous iron, whereas ferritin L plays a role in iron nucleation and protein stability. The H subunit is thought to play a role in rapid detoxification of iron. Orino et al (Orino et al, 2001) in a study using HeLa cells, immoral cells used in scientific researches, reported that synthesis of both subunits of ferritin in HeLa cells exposed to oxidative stress significantly increased ($p<0.05$). They observed that overexpression of either ferritin H or ferritin L reduced the accumulation of ROS in response to oxidant challenge.

In a prospective cohort study to assess the association between breast cancer and total iron and heme iron intake, Kabat et al (Kabat et al, 2007) used data from a large cohort study on Canadian women. In this study, among 49654 women aged 40-59 who were followed for an average of 16.4 years, they identified 2545 incidents of breast cancer. Data from a food frequency questionnaire administrated at baseline was used to calculate total dietary iron and heme iron intake. The results of this study offered no support for an association between iron or heme iron intake and breast cancer risk.

Although several in-vivo and in-vitro and animal studies have shown the effect of iron intake on oxidative stress indicators (Heinecke et al, 1984), (Carrier et al, 2002), there are few human studies in this regard. While some studies have shown negative effects of iron on oxidative stress biomarkers and incidence of some kinds of cancers and the risk of acute myocardial infarction (Tuomainen et al, 1998), (kallianpour et al, 2008), (Bae et al, 2009), other studies have shown the positive role of iron and its related factors such as ferritin, on human body (Orino et al, 2001), (Kabat et al, 2007).

It should be noted that regarding the importance and the specific role of iron in anemic individuals and the necessity of controlling anemia for preventing its adverse outcomes, side effects of iron supplements in developing oxidative stress are usually ignored. Also, most of the studies have focused on the positive effects of fortification programs, and the negative probable aspects of such programs which are effective in controlling and preventing micronutrient deficiencies have been usually ignored.

Also in several studies, the relationship between the level of iron stores and oxidative stress has been shown (Lasheras et al, 2003) (Amirkhizi et al, 2006), (Choi et al, 2008). These studies have indicated that the amount of iron stores and levels of plasma iron have a positive and significant relationship with the increase of fat peroxidation and oxidative stress. In the most recent of these studies, Choi et al in a nested case-control study showed that high iron intake can increase oxidative stress in body which in turn increases the risk of prostate cancer.

Age is also an important factor in antioxidant defence status and there is a positive relationship between age and oxidative stress status (Prashant et al, 2007).

Lack of change in oxidative stress indicators in female subjects can be related to their enhanced physiologic ability in facing oxidative stress. This ability has been shown in the studies of Vina (Vina et al, 2005), Drog (Drog et al, 2002) and Borras (Borras et al, 2003). This ability has been widely studied and different reasons have been presented for it. In the studies of Borras and Vina, it has been stated that the mitochondria of females has a specific ability for producing less amounts of ROS so that males produce ROS two times more than females. In another study, this ability of females has been proposed to be a genetic specification (Vina et al, 2005). Some authors have related this finding to the specific role of estrogen as a strong antioxidant (Ruiz-Larrea et al, 1997) (Borras et al, 2005).

From the results of the two mentioned parts of our study, it can be concluded that although iron intake through flour fortification program did not affect oxidative stress indicators after 32 weeks (8 months), significant changes of

serum iron and also significant changes of some oxidative stress indicators after 64 weeks (16 months) of implementing flour fortification program implies that this program has some side-effects in the long term. In fact, we cannot say flour fortification with iron does not induce oxidative stress among non anemic apparently healthy adults (males) after 64 weeks.

7 CONCLUSION

There are reports on the effect of the supplements of iron on developing oxidative stress and developing cancer. Meanwhile, some studies have not confirmed this effect and even reported the specific mechanisms for controlling surplus iron in the body.

Our field trial results confirmed primary phases of oxidative stress and even it was significant after a longer period of flour fortification in our before and after study. This can be an alarm for the programmers to bear in mind that although using this approach is useful for overcoming anemia, plans to prevent its adverse effects and to control the fortification program at different levels must be devised. In fact, not all the flour should be fortified and non-fortified bread should be always accessible to those who have an appropriate iron status.

Clinical practice and public health policy should be based on reasonably sound evidence that what is being recommended is both safe and effective. Given the results concerning the iron hypothesis to date, there is no doubt about the recommendations. Although further researches, including basic researches and large-scale epidemiologic studies, are needed to fully assess the association between iron status in flour fortification program and the risk of oxidative stress, current data support iron-oxidative stress hypothesis.

7-1 Recommendations

- Reconsidering the level of iron in the flour fortification program.
- Strengthening continuous monitoring of the program in the country
- Not fortifying all the flour that is to be distributed in the society
- Performing a similar study in other age groups
- Performing a similar study with longer period of follow-up
- Giving useful information to the community where flour fortification programs are installed

8 SUMMARY

Despite the advantages of fortifying flour with iron, there are still special concerns regarding the possible adverse effects of the extra iron taken by non-anemic individuals in the general population. Furthermore, there are limited studies regarding the effects of flour fortification with iron on oxidative stress. This study aimed to investigate the effects of fortifying flour with iron on oxidative stress biomarkers and iron status in non-anemic 40- to 65-year-old adults.

This study had two parts: the first part was a randomized field trial and the second part was a before and after study. Among 31 provinces in the Islamic Republic of Iran, Semnan with a low prevalence of anaemia and iron deficiency was selected. Among the cities of this province, Damgan and Semnan were randomized as control and intervention cities, respectively. In the field trial, 393 non-anemic apparently healthy male and female volunteers from Semnan and Damgan were randomly selected. Data gathering was performed in three stages including baseline, after 32 weeks and after 64 weeks. Anthropometric measurements, 3-day 24-hour recall, Food Frequency Questionnaire, and fasting blood samples were collected at the three stages. Following the first stage, flour fortification program started only in Semnan with 30 mg/kg iron as ferrous sulphate and all participants were followed for 32 weeks. Evaluation of oxidative stress using serum levels of malonedialdehyde (MDA), total antioxidant capacity (TAC), super oxide dismutase (SOD), Glutathione Peroxidase (GPx) activity, protein carbonyl, and oxidised-LDL as well as the assessment of iron status and endogenous and dietary antioxidants were done in all three stages.

In the second part of the study (before and after), as flour fortification started in Damgan, we just followed up flour fortification in Semnan to investigate the effect of this program in a longer period (64 weeks).

Results showed that there were no statistically significant changes in iron status and oxidative stress biomarkers after 32 weeks of consuming fortified flour in Semnan compared to Damgan (Field trial).

Results in the before and after study showed that among iron status parameters, serum iron levels in men significantly increased compared to baseline values ($p<0.001$). Among oxidative stress biomarkers, mean TAC significantly decreased ($p<0.001$), and SOD and GPx significantly increased ($p<0.05$) compared to the baseline values only in men. No clinical symptoms of iron overload were observed after 32 and 64 weeks.

Consumption of iron-fortified flour altered certain antioxidant defense biomarkers indicating induced oxidative stress in non-anemic men after 64 weeks. Our findings do not guarantee the safety of flour fortification with 30 mg/kg of iron as a community-based approach to control iron deficiency in non-anemic healthy individuals. Regular amounts of daily flour consumption, burden of iron deficiency, regular monitoring, and the safe amount of iron to be added to flour must be taken into consideration before implementing iron fortification in a population.

9 ZUSAMMENFASSUNG

Trotz der Vorteile der Eisenanreicherung von Mehl, gibt es Bedenken bezüglich möglicher nachteiliger Effekte einer zusätzlichen Eisenaufnahme bei nicht-anämischen Individuen in der allgemeinen Bevölkerung. Außerdem gibt es nur wenige Studien die sich mit den Auswirkungen einer Eisenanreicherung des Mehls auf den oxidativen Stress befassten. Ziel der Studie war die Erforschung des Effekts einer Anreicherung von Mehl mit Eisen auf den Eisenstatus und auf Biomarker des oxidativen Stress bei nicht-anämischen Erwachsenen im Alter zwischen 40 und 65 Jahren.

Die Studie gliedert sich in zwei Abschnitte; der erste Teil war ein randomisierter Feldversuch und der zweite Teil beschreibt die Situation vor und nach der Intervention. Aus 31 Provinzen der islamischen Republik Iran wurde die Provinz Semnan ausgewählt, in der niedrige Prävalenzen für Anämie und Eisenmangel vorherrschen. Aus den Städten dieser Provinz wurden Damgan als Kontroll- und Semnan als Interventionsstadt randomisiert ausgewählt. Für den Feldversuch wurden 393 gesunde Freiwillige beider Geschlechter aus den Städten Semnan und Damgan randomisiert ausgewählt. Die Datenerfassung erfolgte an drei Zeitpunkten: zu Beginn, nach 32 Wochen und nach 64 Wochen. Dabei wurden jeweils anthropometrische Messungen, 24-h recalls über 3 Tage, Food Frequency Questionnaires und Nüchternblutproben erhoben. Nach der ersten Messung wurde die Anreicherung in Form von Eisen(II)-sulfat ausschließlich in Semnan gestartet. Zu allen drei Zeitpunkten wurden der Eisenstatus und der oxidative Stress anhand der Serumlevels von MDA, TAC, SOD, GPx, Proteincarbonyl und oxidiertem LDL ermittelt.

Im zweiten Teil der Studie (davor und danach), wurde die in Semnan durchgeführte Anreicherung beobachtet um deren Langzeiteffekte (64 Wochen) zu erforschen.

Die Ergebnisse zeigten, dass es in Semnan 32 Wochen nach dem Konsum von angereichertem Mehl, verglichen mit Damgan keine statistisch signifikanten Unterschiede bezüglich Eisenstatus und Biomarkern für den oxidativen Stress gab.

Ein vorher-nachher-Vergleich zeigte für die Eisenlevels im Serum bei Männern einen signifikanten Anstieg ($p<0.001$). Unter den Biomarkern für den oxidativen Stress nahm TAC signifikant ab ($p<0.001$), während SOD und GPx, verglichen mit der Basismessung, bei Männern signifikant zunahmen ($p<0.05$). Weder nach 32, noch nach 64 Wochen traten klinische Symptome einer Überbelastung mit Eisen auf.

Der Konsum von eisenangereichertem Mehl veränderte bestimmte Biomarker der antioxidativen Abwehr die ausdrücken, dass es bei nicht-amämischen Männern nach 64 Wochen zu einem oxidativen Stress kam. Die gewonnenen Erkenntnisse unterstützen den Ansatz einer populationsbezogenen Eisenanreicherung mit 30 mg/kg um einen Eisenmangel bei nicht-anämischen gesunden Personen vorzubeugen nicht. Täglicher, ausreichender Konsum von Eisen, das Vorherrschen von Eisenmangel, regelmäßiges Monitoring und die sichere Menge die dem Mehl hinzugefügt werden soll, müssen in Betracht gezogen werden, bevor eine Eisenanreicherung auf Bevölkerungsebene realisiert wird.

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Appendices

NUTRITION DEPARTMENT, UNIVERSITY OF VIENNA, AUSTRIA

The effect of flour fortification with iron on oxidative stress biomarkers and iron status among non anemic adult 40-65 years old

Letter of consent

I **….. ,** declare my agreement for contributing in the researching project of "The effect of flour fortification with iron on oxidative stress biomarkers and iron status…".In this regard, I will answer the questions of researchers precisely and honestly. The goals of this project have been explained to me and I understand that for implementing the biochemical tests of this project, 10-12 milliliter fasting blood will be taken from me in each of the three steps of project, and in each step, my blood sample will be divided in two parts, so that one part will be used for determining anemia (I will be referred to a physician in case of anemia), and the other part will be used for doing complimentary and also oxidative stress tests. Obviously, all of the costs of tests and transporting for samplings will be taken over by researchers and the sponsoring organization of this project. I understand that contributing in this study may have no advantage for me, but it can be useful for enhancing nutritional and health status of my compatriots, and also for national health programming and policy making. In spite of this, I have the right of stopping my cooperation in this project in every time, and I can sue it if any physical or mental damage is made to me. The project administrator guarantee confidentiality of my information and all tests will be free of charge for me, and a copy of all my tests results will be delivered to me too.
Mr./Mrs……..with phone number……is ready to answer my questions about this project during the project time.

Signature and date:

Address of home:
Home phone number:
Address of work place:
Work place number:
Mobile phone number:

NUTRITION DEPARTMENT, UNIVERSITY OF VIENNA, AUSTRIA

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Index
Information form

Date: ID code: Number in cluster: Number of cluster:
Name and surname: City:

1- Age: ….. years
2- Gender…
3- Weight:….Kg
4- Height:…cm
5- BMI:…kg/m^2
6- Literacy: Illiterate ¬ Primary school ¬ Secondary school ¬ Graduated from high school ¬ Academic education ¬
7- Have you consumed iron supplement during last two months?
Yes, regularly ¬ Yes, but irregularly ¬ No ¬
8- Do you consume multivitamin supplements?
Yes, regularly ¬ Yes, but irregularly ¬ No ¬
9- Have you had any surgical operation during last two months? Yes ¬ No ¬
10- Are you going to undertake any surgery during the following year? Yes ¬ No ¬
11- Do you smoke? Yes ¬ No ¬
12- How many cigarettes do you smoke daily? ….
13- How many years have you been smoking? ….
14- Does anybody smoke in your home? Yes ¬ No ¬
15- Have you had any febrile disease during last month? Yes ¬ No ¬
16- Do you have any specific disease (with diagnosis of a physician)?
No ¬ Yes ¬ name of disease……
17- Do you consume alcohol? Yes, regularly ¬ Yes, but irregularly ¬ No¬
18- Amount of alcohol consumption/ day? …..
19-Have you donated blood during last month? Yes ¬ No ¬
20-which drugs do you consume under medical prescription? ....
22-Blood pressure:
First time …..mmHg Second time…… mmHg Third time ….mmHg

Address of home:
Home phone number:
Address of work place:
Work place number:
Mobile phone number:

Name of questioner: **signature:** **date:**

Name of controller: **signature:** **date:**

NUTRITION DEPARTMENT, UNIVERSITY OF VIENNA, AUSTRIA

The effect of flour fortification with iron on oxidative stress biomarkers and iron status among non anemic adult 40-65 years old

24hour recall

Date: ID code: Number in cluster: Number of cluster:

Name and surname: City:

ingestion period	Food	Food components	Index and amount of food
Breakfast			
between-meal foods			
Lunch			
between-meal foods			
Dinner			
between-meal foods			

Name of questioner: **signature:** **date:**

Name of controller: **signature:** **date:**

NUTRITION DEPARTMENT, UNIVERSITY OF VIENNA, AUSTRIA

The effect of flour fortification with iron on oxidative stress biomarkers and iron status among non anemic adult 40-65 years old

Food Frequency Questionnaire (FFQ)

Date: ID code: Number in cluster: Number of cluster:

Name and surname: City:

No.	Food	Never	Consumption per				Index	unit
			day	week	month	year		
1	**Rice**						Spatula(usual, full), Plat	
2	**Barbari bread**						Palm size(number)	
3	**White Bread**						Palm size(number)	
4	**Lavash bread**						Palm size(number	
5	**Sangak bread**						Palm size(number)	
6	**Dry bread of Semnan**						Palm size(number)	
7	**Fatir bread**						Palm size(number)	
8	**Wheat flour**						Glass, bowl, table spoon	
9	**Vermicelli(uncooked)**						Bowl	
10	**Spaghetti**						Spatula(usual, full), Plat	
11	**Beef and lamb**						Piece (30 gr)	
12	**Ground meat**						Table spoon	
13	**Kidney, heart and liver**						Piece(30 gr)	
14	**Chicken with skin**						Leg, chest, arm, neck, all piece(L, M, S)*	
15	**Chicken without skin**						Leg, chest, arm, neck, all piece(L, M, S)*	
16	**Heart, liver and gizzard**						Number(30 gr)	
17	**Egg**						One whole(60 gr)	
18	**Fish**						150 gr	
19	**Canned tuna fish**						Can(200 gr)	

*l= larg size, M= medium size, S= small size

No.	Food	Never	Consumption per				Index	unit
			day	week	month	year		
20	Sussages						One slice (30 gr)	
21	kielbasa						One slice (10gr)	
22	Hamburger						Number(90gr)	
23	Pasteurized milk						Glass, cup	
24	Raw milk						Glass, cup	
25	Pasteurized cheese						Matchbox size(30gr)	
26	Rural cheese						Matchbox size(30gr)	
27	Medium fat yoghurt						Bowl, glass	
28	High fat yoghurt						Bowl, glass	
29	Low fat yoghurt						Bowl, glass	
30	Doogh (yoghurt drink)						Bowl, glass	
31	Pasteurized curds						Table spoon	
32	Navy been						Bowl, glass	
33	Kidney been						Bowl, glass	
34	Wax bean						Bowl, glass	
35	Pea						Bowl, glass	
36	Split pea						Bowl, glass	
37	Soy bean						Bowl, glass	
38	lentil						Bowl, glass	
39	Butter						Matchbox size	
40	Cream						Tea spoon, table spoon	
41	Hydrogenated vegetable oil						Table spoon (semi full, full)	
42	Vegetable oil						Table spoon	
43	Mayonnaise						Table spoon	
44	Olive oil						Table spoon	
45	Fried potato						Chip, ring, cube	
46	Water cooked potato						L, M, S*	
47	Uncooked tomato						L, M, S*	

No.	Food	Never	Consumption per				Index	unit
			day	week	month	year		
48	**Cooked tomato**						L, M, S*	
49	**Tomato paste**						Table spoon	
50	**Eggplant**						L, M, S*	
51	**Uncooked onion**						L, M, S*	
52	**Cooked onion**						L, M, S*	
53	**Leafy vegetables**						Bowl (L, M, S*)	
54	**Green bean**						Bowl, glass	
55	**Green pea**						Bowl, glass	
56	**Fava bean**						Bowl, glass	
57	**lettuce**						Glass, leaf	
58	**Squash**						Slice	
59	**Cooked pumpkin**						Slice	
60	**Cabbage leaf(chopped)**						bowl	
61	**Mushroom**						L, M, S*	
62	**Green pepper**						L, M, S*	
63	**Sweet pepper**						L, M, S*	
64	**Uncooked carrot**						L, M, S*	
65	**Cooked carrot**						L, M, S*	
66	**Uncooked garlic (cloves)**						L, M, S*	
67	**watermelon**						Slice(L, M, S*)	
68	**Melon**						L, M, S*	
69	**Cucumber**						L, M, S*	
70	**Orange**						L, M, S*	
71	**Tangin and other citrus**						L, M, S*	
72	**Grape**						Glass	
73	**Apple with peeling**						L, M, S*	

*l= larg size, M= medium size, S= small size

No.	Food	Never	Consumption per				Index	unit
			day	week	month	year		
74	Apple without peeling						L, M, S*	
75	Apricot						Number(30gr)	
76	Plum						L, M, S*	
77	Cherry/sour cherry						Glass(L, M, S*)	
78	Peach						L, M, S*	
79	Pear						L, M, S*	
80	Dates						Number(L, M, S*)	
81	Banana						L, M, S*	
82	Pomegranate						L, M, S*	
83	Raisin						Bowl, cup, table spoon	
84	Barberry						Bowl, cup, table spoon	
85	Dried mulberry						20 medium size (17gr)	
86	Dried fig						Number	
87	Dried prune						Number	
88	Dried apricot						Number	
89	Peanut						Bowl(with or without shell)	
90	walnut						L, M, S*	
91	Pistachio						Bowl, number	
92	Cube sugar						cube	
93	Sugar						Tea spoon, table spoon	
94	Honey						Tea spoon, table spoon	
95	jam						Tea spoon, table spoon	
96	Halwa						Tea spoon, table spoon	
97	candy						number	
98	Chocolate						number	
99	Sponge cake						40 gr	

No.	Food	Never	Consumption per				Index	unit
			day	week	month	year		
100	Fruit juice						Milliliter	
101	Conserved fruit						Glass	
102	Carbonated drink						Bottle, glass	
103	Tea						Glass, cup	

Name of questioner: **signature:** **date:**

Name of controller: **signature:** **date:**

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

SHORT LAST 7 DAYS FORMAT

For use with Young and Middle-aged Adults (15-69 years)

I am going to ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Now, think about all the *vigorous* activities which take *hard physical effort* that you did in the last 7 days. Vigorous activities make you breathe much harder than normal and may include heavy lifting, digging, aerobics, or fast bicycling. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities?

   _____ Days per week [VDAY; Range 0-7, 8,9]

   8. Don't Know/Not Sure

   9. Refused

[**Interviewer clarification**: Think only about those physical activities that you do for at least 10 minutes at a time.]

[**Interviewer note**: If respondent answers zero, refuses or does not know, skip to Question 3]

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   __ __ Hours per day [VDHRS; Range: 0-16]

   __ __ __ Minutes per day [VDMIN; Range: 0-960, 998, 999]

   998. Don't Know/Not Sure

   999. Refused

   [**Interviewer clarification**: Think only about those physical activities you do for at least 10 minutes at a time.]

[**Interviewer probe**: An average time for one of the days on which you do vigorous activity is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "How much time in total would you spend **over the last 7 days** doing vigorous physical activities?"

__ __ Hours per week [VWHRS; Range: 0-112]

__ __ __ __Minutes per week [VWMIN; Range: 0-6720, 9998, 9999]

9998. Don't Know/Not Sure

9999. Refused

Now think about activities which take moderate physical effort that you did in the last 7 days. Moderate physical activities make you breathe somewhat harder than normal and may include carrying light loads, bicycling at a regular pace, or doubles tennis. Do not include walking. Again, think about only those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities?

____ Days per week [MDAY; Range: 0-7, 8, 9]

8. Don't Know/Not Sure

9. Refused

[**Interviewer clarification**: Think only about those physical activities that you do for at least 10 minutes at a time]

[**Interviewer Note**: *If respondent answers zero*, refuses or does not know, skip to Question 5]

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

__ __ Hours per day [MDHRS; Range: 0-16]

__ __ __ Minutes per day [MDMIN; Range: 0-960, 998, 999]

998. Don't Know/Not Sure

999. Refused

[**Interviewer clarification**: Think only about those physical activities that you do for at least 10 minutes at a time.]

[**Interviewer probe**: An average time for one of the days on which you do moderate activity is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, or includes time spent in multiple jobs, ask: "What is the total amount of time you spent over the **last 7 days** doing moderate physical activities?"

__ __ __ Hours per week [MWHRS; Range: 0-112]

__ __ __ __Minutes per week [MWMIN; Range: 0-6720, 9998, 9999]

9998. Don't Know/Not Sure

9999. Refused

Now think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

____ Days per week [WDAY; Range: 0-7, 8, 9]

8. Don't Know/Not Sure

9. Refused

[**Interviewer clarification**: Think only about the walking that you do for at least 10 minutes at a time.]

[**Interviewer Note**: *If respondent answers zero*, refuses or does not know, skip to Question 7]

6. How much time did you usually spend **walking** on one of those days?

__ __ Hours per day [WDHRS; Range: 0-16]

__ __ __ Minutes per day [WDMIN; Range: 0-960, 998, 999]

998. Don't Know/Not Sure

999. Refused

[**Interviewer probe**: An average time for one of the days on which you walk is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "What is the total amount of time you spent walking over **the last 7 days**?"

__ __ __ Hours per week [WWHRS; Range: 0-112]

__ __ __ __Minutes per week [WWMIN; Range: 0-6720, 9998, 9999]

9998. Don't Know/Not Sure

9999.Refused

Now think about the time you spent sitting on week days during the last 7 days. Include time spent at work, at home, while doing course work, and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television.

7. During the last 7 days, how much time did you usually spend ***sitting*** on a **week day**?

__ __ Hours per weekday [SDHRS; 0-16]

__ __ __ Minutes per weekday [SDMIN; Range: 0-960, 998, 999]

998. Don't Know/Not Sure

999. Refused

[**Interviewer clarification**: Include time spent lying down (awake) as well as sitting]

[**Interviewer probe**: An average time per day spent sitting is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "What is the total amount of time you spent *sitting* last **Wednesday**?"

__ __ Hours on Wednesday [SWHRS; Range 0-16]

__ __ __ Minutes on Wednesday [SWMIN; Range: 0-960, 998, 999]

998. Don't Know/Not Sure

999. Refused

Curriculum Vitae

PERSONAL INFORMATION

Name:	**MSc. Hamed Pouraram**
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EDUCATION

- PhD Student in Nutritional Sciences,

  Austria, University of Vienna 2006 – at present
- MSc in Nutrition Sciences,

  Shahead Beheshti University of Medical Science,

  Tehran, Iran 2000 - 2003
- BSc in Nutrition, Tabriz,

  University of Medical Science, Tabriz 1991-1995

WORK EXPERIENCE

- Senior Expert of Nutrition Department 2001- at present
- Flour fortification manager in MOH, IRAN, 2003- 2008
- Head of Scientific Group for Enhancing the Quality of Bread, 2003-2007
- National Executive Consultant for Flour Fortification Program, Iran, 2002-2003
- Fortification officer of Nutrition Dep. 2000- 2001
- Staff member of the Nutrition Dep. 1999-2000
- Nutrition Expert in the Health Center of Astane Ashrafieh (City) 1997-1999

RESEARCH PROJECTS

- Effect of Folic Acid in Flour Fortification Program in Busher and Golestan, IRAN, 2009, MOH.
- Impact of Flour Fortification Program , 2007, MOH
- Assessment of New Premix in Flour Fortification Program, Dailam (City), 2005, MOH&ME, DSM.
- Anthropometric and Nutritional Index Survey ANIS 2 Fars & Kohkiloie, Iran, 2004, MOH&ME and UNICEF.
- Prevalence of Goiter on School Children, Kerman, Iran, 2002, MOH and UNICEF.
- KAP Survey about Flour Fortification Program in 3 Provinces in Iran, 2004, MOH&ME and Epidemiological Council in Iran (ECI).
- Midterm Evaluation of Flour Fortification in Bushehr. 2003, MOH & WORLD BANK.
- Micronutrient Survey in Iran (NIMS), 2001, MOH, NNFTRI, UNICEF.
- Nutritional Support for Children in Poor families, 1999, MOH,EMAM ASSIST Committee.
- Assessment of UNICEF Electronic Scale, 1999, UNICEF.
- DHS in Iran, 1999, MOH, UNFPA.

PUBLICATION

- **Pouraram H**, Elmadfa I, Siassi F, Dorosty AR, Heshmat R, Abtahi M. Oxidative stress following flour fortification program among non-anemic adults: Baseline data. Annals of Nutrition and Metabolism, 2010 (Accepted).

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